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Quantitative analysis of amylopectin unit chains by means of high-performance anion-exchange chromatography with pulsed amperometric detection

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Abstract

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) is a widely used technique to study the chain length distribution of amylopectin. The chromatograms, however, do not directly reflect this distribution, since the PAD response changes with the degree of polymerization. In this study, waxy maize starch was debranched and fractionated on a Bio-Gel P-6 column and the response factors for maltosaccharides with DP 3–65 were determined. The detector response per μg glucan chains was shown to decrease considerably for DP 3–7 while the curve leveled out for DP larger than 15. © 1998 Elsevier Science B.V.

Keywords: Amylopectin; Starch; Saccharides

1. Introduction

The physicochemical properties of starch are to a large extent governed by the ratio of amylose to amylopectin and by the distribution of unit chains in these molecules. Starches isolated from various botanical sources differ in structure and have therefore different functional properties when used as raw material, e.g. in the food and paper industry [1,2]. Recently, there has been an increased interest in starch research, partly because of new knowledge of the mechanism of starch synthesis and partly due to the use of biotechnology in plant breeding which has increased the possibility of developing starches with modified properties [3]. Size-exclusion chromatography (SEC) is a widely used technique after debranching of the starch that gives a good overall view of the amylopectin chain length distribution. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD), however, provides more details such as the amount of the individual unit glucan chains. Improvements of column materials have made it possible to separate individual maltosaccharides with a degree of polymerization (DP) up to 80 with high resolution [4]. The basis for the anion-exchange process in starch analysis is competitive binding of weakly ionized glucan chains and ions in the eluent to the oppositely charged column. Changing pH to elute the sample is not appropriate since a high pH is required to ionize the molecules and instead an ionic strength gradient with sodium acetate is used. The efficiency of different ions as compatible counter ions has been investigated and hydroxide ions were found to be the

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weakest and sulphate ions the strongest pushing agents while carbonate, nitrate and sulphate ions were found to reduce the loading capacity of the column because of their high affinity for the anion-exchange resin [5–7]. However, Rocklin and Pohl [7] found acetate to be the preferred ion as an eluent additive as it has similar affinity for the anion-exchange resin to that of hydroxide.

For quantitative analyses it is necessary to know the relative detector response (RDR) of the individual polysaccharides separated by HPAEC. Several investigators have studied the PAD response for maltosaccharides with low degree of polymerization (DP 2–17) [8,9]. They found that PAD showed a different response for chains of different length. Therefore, the peak area in a HPAEC chromatogram does not directly reflect the chain length distribution. The different detector responses must be compensated for by using a relative response factor for each chain length.

The aims of this investigation were to optimize the chromatographic conditions for HPAEC and to determine the relative detector responses especially for longer maltosaccharides in order to accurately determine the chain length distribution in amylopectin.

2. Experimental

2.1. Materials

Wheat, potato, pea and waxy maize starch were obtained from Lyckeby Stärkelsen (Kristianstad, Sweden). Maltotriose (Sigma, St. Louis, MO, USA), maltopentaose and maltoheptaose (Boehringer Mannheim, Mannheim, Germany) were used as standards. Sodium hydroxide solution (analytical-reagent grade, 50%) was purchased from Baker (Deventer, Netherlands) and sodium acetate (analytical-reagent grade) from Fluka (Buchs, Switzerland). Isoamylase (EC 3.2.1.68, crystal, from *Pseudomonas amyloderamosa*, 71 000 U/mg) was obtained from Hayashibara Biochemical Labs. (Okayama, Japan), amyloglucosidase (EC 3.2.1.3 from *Aspergillus niger*, 36 U/mg) from Megazyme (Bray, Ireland) and Merckotest Glucose (Article No. 14365) from Merck (Darmstadt, Germany).

2.2. Isolation and debranching of amylopectin

Starch from wheat, pea and potato was dissolved in 0.1 *M* NaOH and the amylopectin isolated and freeze-dried [10]. Waxy maize starch or isolated amylopectin (16 mg) was debranched with isoamylase [10,11].

2.3. Ion-exchange chromatography

The chromatography was performed on a Dionex DX 500 instrument (Sunnyvale, CA, USA) equipped with a PAD system (ED 40). Samples (20 μl) with a concentration of 0.2–1 mg debranched amylopectin per ml were injected, without prior filtration, onto a CarboPac PA-100 anion-exchange column (250×4 mm) in combination with a CarboPac PA-100 guard column via an autosampler (Spectra-Physics, Fremont, CA, USA). The potentials and time periods for the pulsed amperometric detection were: E_1, +0.05 V for 480 ms; E_2, +0.60 V for 120 ms; E_3, −0.80 V for 300 ms. The flow-rate was 1.0 ml/min and the acetate gradient system included two eluents. Eluent A was 150 m*M* sodium hydroxide and eluent B was 150 m*M* sodium hydroxide containing 500 m*M* sodium acetate. The gradient was optimized using a full factorial design, covering the shaded area in Fig. 1, in order to determine the appropriate level of eluent B at 0, 5 and 40 min. The levels were 20 and 40% B at 0 min, 40 and 60% B at 5 min and 60 and 80% B at 40 min. The system was equilibrated with the initial eluent for 15 min before each run. The optimal gradient found was: 0–5 min, linear gradient from 34% to 45% eluent B; 5–40 min, linear gradient to 60% eluent B, this part of the gradient being elongated to 67% eluent B at 55 min; and 55–80 min, linear gradient to 90% eluent B.

2.4. Isolation of amylopectin unit chain fractions

Debranched waxy maize starch was fractionated by gel permeation chromatography on a Bio-Gel P-6 column (75×1.6 cm) (Bio-Rad, Richmond, CA, USA) using water as eluent at a flow-rate of 0.4 ml/min. The sample was monitored by a refractive index (RI) detector (Model R-403, Waters, Milford, MA, USA) and 1.6 ml-fractions were collected. An aliquot from the fractions was diluted 1:1 with water

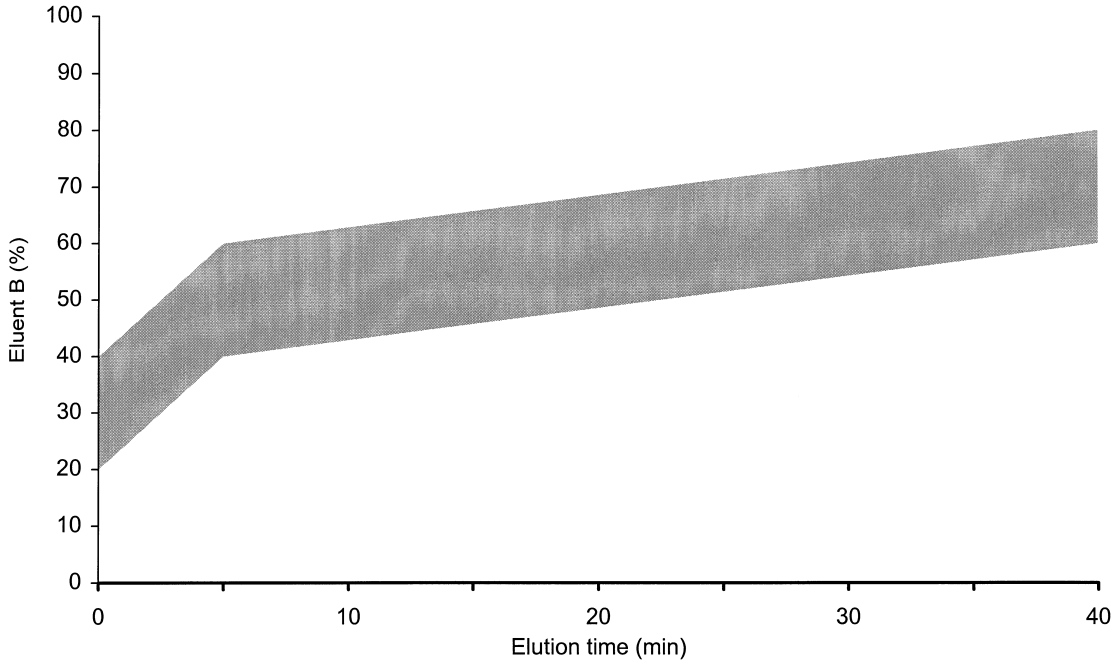

Fig. 1. The area represents the gradients covered in the full factorial design for optimization of the chromatographic conditions for HPAEC.

and 20 μl injected without filtration onto the HPAEC instrument. The content of glucose residues in each unit chain fraction was determined essentially according to Åman et al. [12]. 500 μl of each fraction was incubated with 500 μl 400 m*M* acetate buffer containing 3 U/ml amyloglucosidase for 4 h at 60°C in a shaking water bath. Two ml of Merckotest Glucose was added and the absorbance was measured at 510 nm.

2.5. Determination of the detector response

Twenty μl maltotriose, maltopentaose or maltoheptaose solution (0.01 mg/ml) as well as the unit chain fractions were subjected to HPAEC as described above. The average DP for each unit chain fraction (f) was calculated as:

$$\overline{\mathrm{DP}_f} = \frac{\sum_{i=1}^{n}(A_i N_i)}{\sum_{i=1}^{n} A_i} \quad (1)$$

where n = number of peaks, A_i = peak area and N_i = DP for peak i. The retention time for maltoheptaose was used to determine the DP for the peaks in the chromatograms of the debranched amylopectin. The detector response for each fraction was calculated as:

$$R_f = \frac{A_f}{W_f} \quad (2)$$

where A_f = total peak area and W_f the amount of glucan chains for fraction f.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

Good chromatographic conditions are essential for reliable quantitative HPAEC analyses. Therefore, the gradient was optimized by means of a full factorial design (Table 1). The purpose was to find the best elution gradient that comprises good resolution at the beginning as well as at the end of a chromatographic run and elution of as long maltosaccharides as

Table 1
Linear chromatographic gradient, retention times and resolution for designed experiments and amylopectin samples

		Concentration of eluent B (%)			Retention time (min)				Resolution[a]			
	Run order	0 min	5 min	40 min	DP 8	DP 15	DP 25	DP 30	DP 8/9	DP 15/16	DP 25/26	DP 30/31
Designed experiments												
1	6	20	40	60	8.0	14.7	31.5	38.8	2.4	2.3	1.1	0.8
2	11	40	40	60	5.0	12.6	31.2	38.8	2.0	2.8	1.1	0.9
3	7	20	60	60	6.2	7.7	9.9	12.2	1.6	1.0	0.7	0.5
4	2	40	60	60	4.6	6.6	9.5	12.1	1.4	1.1	1.0	1.2
5	4	20	40	80	8.0	13.5	22.7	26.2	2.4	2.2	1.5	1.2
6	10	40	40	80	5.1	12.1	22.6	26.2	1.8	2.2	1.2	1.1
7	5	20	60	80	6.2	7.7	9.7	11.4	1.5	0.8	0.7	0.8
8	8	40	60	80	4.6	6.6	10.8	13.4	1.3	1.1	1.5	1.1
9	3	30	50	70	6.0	8.9	16.6	22.1	2.1	1.5	1.3	0.8
10	9	30	50	70	5.9	8.8	16.5	22.0	2.1	1.5	1.1	0.8
11	1	30	50	70	5.9	8.9	16.7	22.2	2.8	1.7	1.5	1.1
Amylopectin samples												
Waxy maize		34	45	60	4.8	10.3	24.3	32.0	2.0	1.8	1.7	1.4
Wheat		34	45	60	5.1	11.2	27.0	35.2	2.1	1.8	1.7	1.5
Potato		34	45	60	5.1	11.4	27.4	35.5	2.0	1.8	1.7	1.5
Pea		34	45	60	5.1	11.2	27.0	35.2	2.0	1.6	1.7	1.5

[a] DP 8/9 denotes the resolution between peaks of DP 8 and DP 9, expressed as the difference in retention time divided by average peak width.

possible. Three points (0, 5 and 40 min) were empirically identified as crucial phases for the elution pattern and were set as factors in the full factorial design. Waxy maize starch was debranched and run using the eleven designed gradients in a randomized order. A large experimental area (Fig. 1) was covered using this approach. The peaks representing DP 8, 15, 25 and 30 were chosen in order to reveal how retention and resolution were influenced by the gradient. A good reproducibility was indicated by the fact that the results for the three centre point (9, 10 and 11) runs did not differ significantly (Table 1). The retention time for DP 8 was highly dependent on the level of eluent B at 0 min; 6.2–8.0 min with 20% and 4.6–5.1 min with 40% eluent B. The retention times for DP 15–30 were mainly influenced by the gradient level at 5 min. Shorter retention times for DP 25 and 30 were observed with 60% eluent B at 5 min regardless of whether the level at 40 min was 60 or 80%. The resolutions were correlated to the level of eluent B at 5 min. With 40% eluent B at 5 min the resolution was higher for DP 8/9 and 15/16 than with 60% eluent B. The level of eluent B at 40 min, however, affected the resolution of DP 25/26 but the effect on DP 30/31 was less pronounced. There was no relationship between retention time and resolution.

Gradients 5 and 6 and the centre points resulted in the best resolutions. By comparing the results from the eleven chromatograms a gradient was created that separated the unit chains of debranched amylopectin with satisfactory resolution and retention times. These conditions were verified by the good resolution values obtained for wheat, potato and pea amylopectin.

3.2. Isolation of unit chain fractions

It is difficult to obtain standards with high DP. Therefore, debranched waxy maize starch was fractionated on Bio-Gel P-6 in order to obtain fractions with a series of different average DP. The fractionation was followed by quantitative determination of glucose residues in each fraction. Every second fraction was analysed by HPAEC and the DP range was determined by the chromatographic profile (Fig. 2). The higher the average DP, the more peaks in the chromatograms because of poorer resolution on the Bio-Gel P-6 column for longer glucan chains. The DP-interval in each fraction was considered narrow

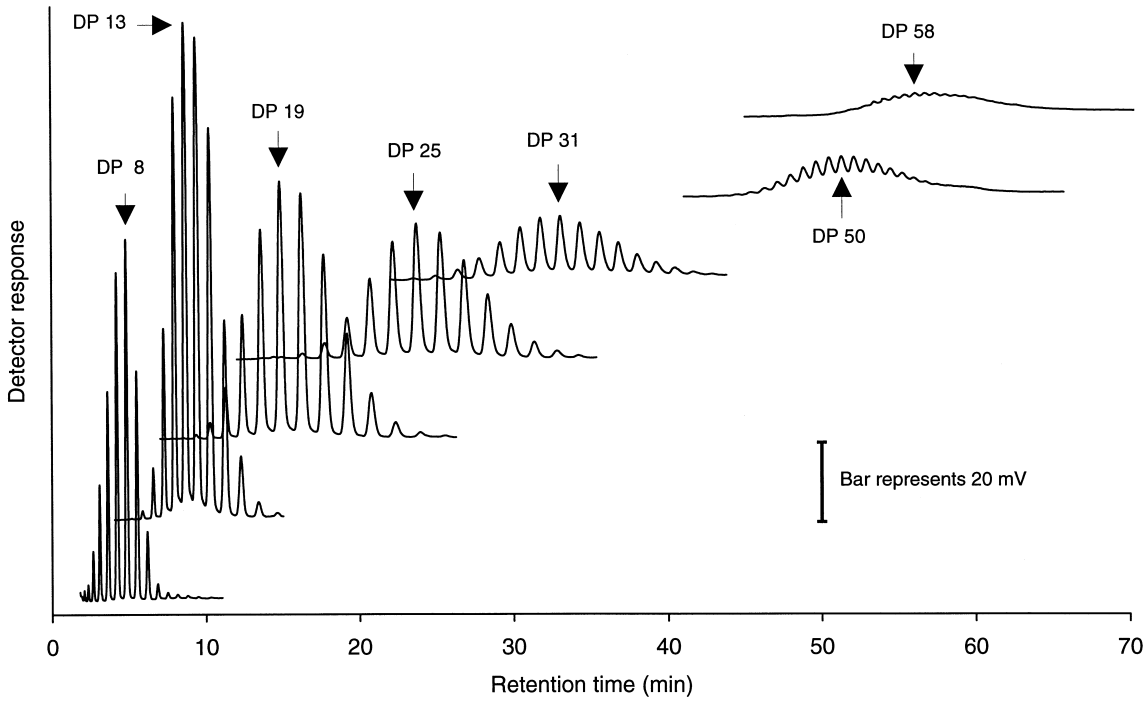

Fig. 2. HPAEC elution profiles of seven selected fractions of debranched waxy maize.

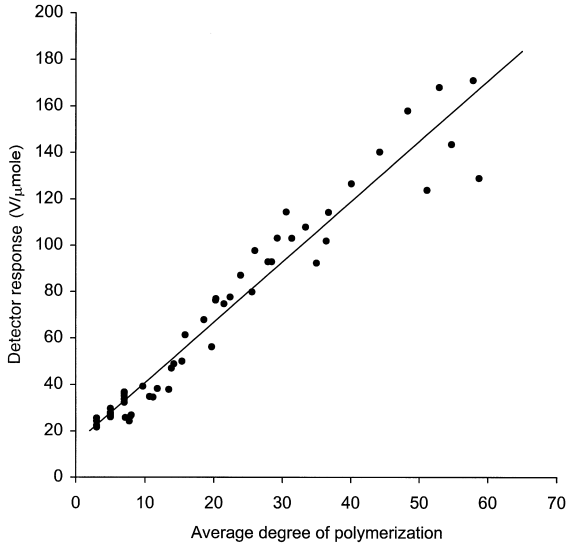

Fig. 3. The relationship between the detector response of PAD per μmole glucan chain and the degree of polymerization.

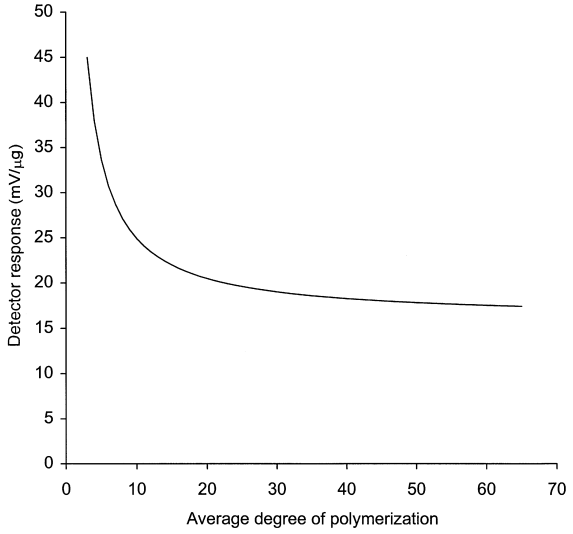

Fig. 4. The relationship between the detector response of PAD per μg glucan chains and the degree of polymerization.

enough to be used as calibration standards in this investigation, however.

3.3. Stability of the unit chain fractions

The HPAEC analysis of the selected unit chain fractions took two days during which the samples were stored at room temperature. It was therefore necessary to study their stability over time. There was also a risk of precipitation for the longer unit chain fractions. Fractions at low and at high DP were therefore selected for analysis. Sample injection into the HPAEC and enzymatic analysis of glucose residues were carried out directly after the gel filtration and repeated the two following days. Neither peak area nor the number of peaks in the chromatograms differed significantly over the consecutive days. Furthermore, the deviations of the glucan concentration over the three days were not significantly larger than those obtained between replicates, indicating a satisfactory stability for the unit chain fractions.

3.4. Determination of the detector response

The average degree of polymerization was calculated for each unit chain fraction. The estimated DP values for the fractions with the longest unit chains varied 1–3 units between replicates. The concentration of glucose residues in the unit chain fractions was expressed as mole/ml and was used for calculating the response factor. There was a linear relationship between the detector response per mole glucan chains and the degree of polymerization (Fig. 3). This is in agreement with earlier studies of Koizumi et al. [8] who found a proportional increase of the relative detector responses, on a molar basis, for maltosaccharides with DP 6–17. Similarly, Shi and Seib [9] found the molar responses of the PAD detector to increase with chain length from DP 2–15. Using the regression line in Fig. 3, the relationship between the response factors on weight basis and DP was calculated (Fig. 4). A steep decline in the detector response per μg glucan chains for DP 3 to 7 was observed while the curve leveled out for maltosaccharides of DP higher than 15. Koizumi et al. [8] found the relationship between RDR per HCOH and DP to be similar to our results, but suggested a decreasing detector response from DP 14 to 17. Timmermans et al. [13] found a similar pattern when studying the sensitivity of PAD for inulin with DP 2–5 and 10–17. The influence of the individual hydroxyl groups of the glucose residues on the retention time and relative response factor have previously been investigated. A possible explanation for the declining RDR is that the ratio of the most acidic OH group on the reducing end to other hydroxyl groups in a molecule is larger for short chains than for the longer ones, leading to a more effective oxidation at the Au electrode for the former [14,15]. Koizumi et al. [8] suggested that the random coil formation of glucans with increasing molecular mass leads to an increase in undetectable OH groups and lower detector response. Our goal, though, was not to understand the mechanism of the RDR, but to obtain a calibration of the HPAEC–PAD method that is applicable on debranched amylopectin. The earlier investigators have focused on the detector response for glucan chains in the low DP range. In this study, however, the RDR for the low DP range as well as the high DP range was investigated, which is necessary for the quantification of the unit chains in debranched amylopectin.

3.5. Analysis of amylopectin samples

The optimized gradient was applied when analysing waxy maize starch and isolated amylopectin from wheat, potato and pea. The relative peak areas and the relative amounts of unit chains were calculated and the profiles obtained were slightly different (Fig. 5). Generally, relative amount versus DP gives a more pronounced polymodal chain length distribution. The most important difference was for the short chains, which is explained by the relatively high detector response for those glucans. Maltosaccharides of DP$>$65 may be measured with other techniques such as SEC. In summary, distributions expressed as relative area may give a misleading impression while a more accurate distribution of amylopectin chains with DP 3–65 can be obtained by using response factors.

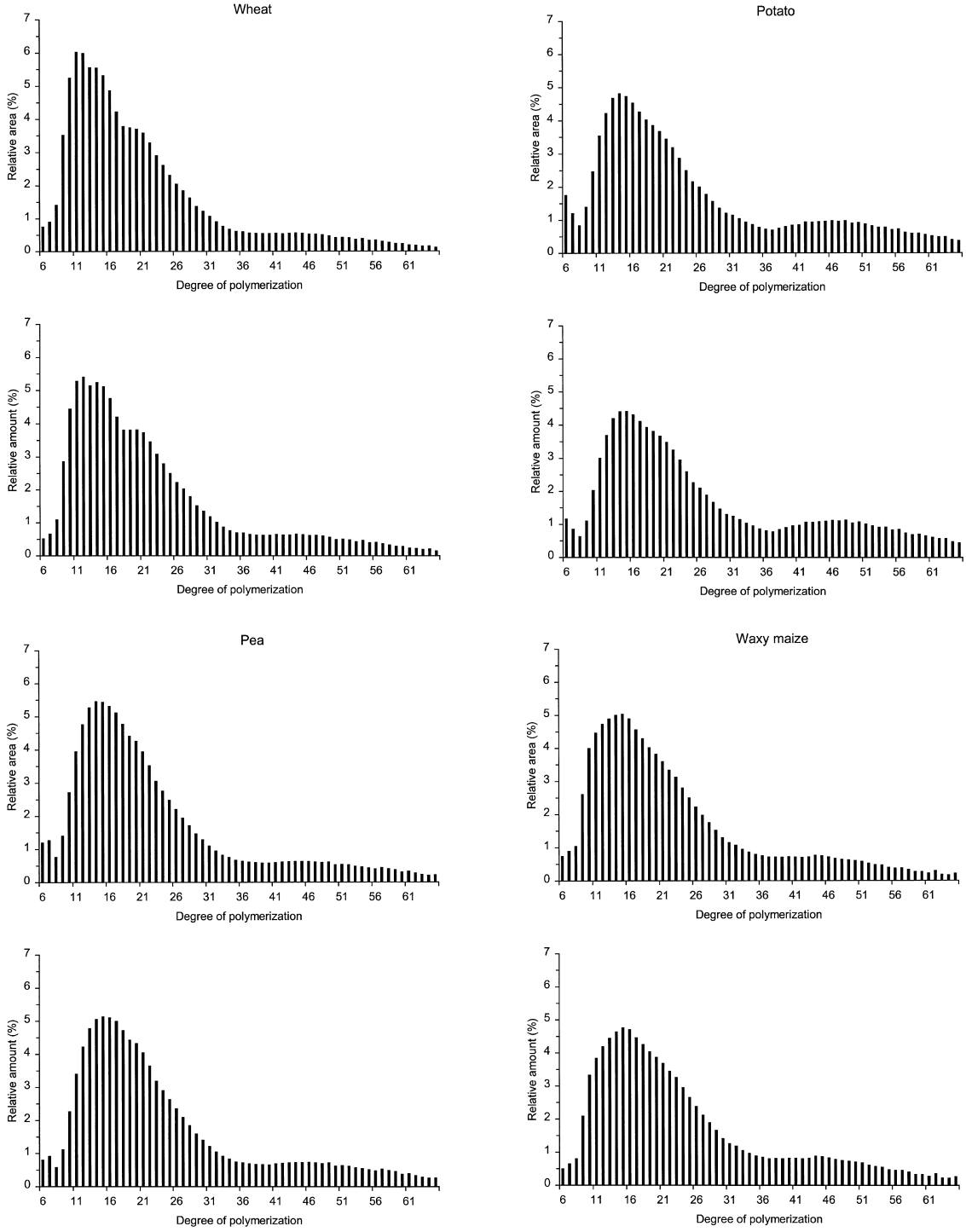

Fig. 5. Bar graphs showing the chain length distribution of wheat, potato, pea and waxy maize amylopectin on a relative area and relative amount basis.

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