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Amylose determination in genetically modified starches

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Abstract

The amylose contents of starches from various botanical origins (potato, smooth pea, wrinkled pea, wheat, maize) and from maize mutants [*waxy* (*wx*), *amylose extender* (*ae*), *dull* (*du*), *sugary*-2 (*su*2), *aewx*, *aedu*, *dusu*2 and *wxdu*] were determined by size exclusion chromatography (SEC), iodine-binding capacity (IBC) measurements, differential scanning calorimetry (DSC) and complexation with concanavalin A. SEC (with a 2.6 × 200 cm column) on HW75 S gel was used as the reference method for analyzing the macromolecular composition of starches. Variations in the fine structure of amylopectin affected its reactivity in classical methods such as IBC and were probably responsible for erroneous values in determinations when this polymer was the only starch component studied. When starches were composed of two macromolecules, all methods gave similar results, but with some discrepancies in DSC. The elution volume for a third class of α-glucans detected in some maize mutant starches was between that of amylopectin and amylose. Only SEC gave accurate results in this case since all other tested methods showed higher apparent amylose contents.

Keywords: Starch; Amylose; Iodine-binding capacity; Concanavalin A; Differential scanning calorimetry; Size exclusion chromatography

1. Introduction

Starch granules are mainly composed of two macromolecular polymers of α,D-glucose, amylose and amylopectin (Banks & Greenwood, 1975). Amylose is a linear glucan with α,1–4 glycosidic linkages and limited branching that produces few non-reducing end-groups. Its molecular weight is between 10^5 and 10^6 g mol^{-1} (Roger, Tran, Lesec & Colonna, 1996). Conversely, amylopectin is a highly branched polymer composed of short linear chains [degree of polymerization (dp) = 15] branched on longer chains (dp = 45) by α,1–6 linkages. Its molecular weight is greater than 10^8 g mol^{-1} (Roger, Bello-Perez & Colonna, 1999). Depending on botanical origin, the structural features of amylose and amylopectin differ. The proportion of amylose in starch ranges from 0 in *waxy* (*wx*) maize starch up to 70–80% in *rugosus* (*r*) pea starch (i.e. wrinkled pea). Amylose content is known to influence both nutritional and technological properties such as susceptibility to enzymatic hydrolysis, gelling and pasting behavior (Whistler, BeMiller & Paschall, 1984).

Many studies have attempted to determine exact amylose content since the pioneering work of Larson, Gilles and Jennes (1953), Adkins and Greenwood (1966) and, Banks and Greenwood (1975). Methods have been based on structural or functional differences between the two theoretical macromolecules present in starch. Amylose content is commonly measured by iodine-binding procedures, whether potentiometric (Banks & Greenwood, 1975), amperometric (Larson et al., 1953), or spectrophotometric (Morrison & Laignelet, 1983). These procedures are based on the capacity of amylose to form helical inclusion complexes with iodine, which display a blue color characterized by a maximum absorption wavelength (λ_{max}) above 620 nm. The iodine-binding capacity (IBC) of pure amylose is around 20 mg per 100 mg (Banks & Greenwood, 1975). However, amylopectin can also form complexes, though more weakly (IBC ≈ 1 mg per 100 mg), and develop a red color characterized by a λ_{max} of around 540 nm. Phospholipids and fatty acids, which can be complexed, must be extracted before measurements in order to avoid underestimations. The calorimetric method also takes this capacity into account (Kugimiya & Donovan, 1981; Sievert & Holm, 1993) since it is based on measurement of the enthalpy of the melting or formation of amylose–lipid complexes after complete dispersion of the granular structure in the presence of an excess of phosphilipids or monoglycerides. No defatting is necessary, and this method can be applied to complex mixtures such as flours and semolinas (Mestres, Matencio, Pons, Yajid & Fliedel, 1996).

Another approach for determining the proportion of amylose is based on the different number of non-reducing end-groups between amylose and amylopectin (Gibson,

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Solah & McCleary, 1997). Lectin concanavalin A (Con A) interacts specifically with α-D-glucosyl units at multiple non-reducing end-groups. In the case of amylopectin, the considerable number of non-reducing end-groups per molecule allows the formation of a precipitate under defined conditions of pH, temperature and ionic strength (Colonna, Biton & Mercier, 1985; Matheson, 1990). After removal of amylopectin by precipitation, amylose content is determined by measuring the amount of carbohydrates in the supernatant.

Size exclusion chromatography (SEC), either on debranched or native starch, has also been used to measure amylose content. This method allows molecules to be separated according to their hydrodynamic radius. With isoamylase–debranched starch, the dp of the constitutive chains has been analyzed on Sepharose CL-4B, 6B (Sargeant, 1982) or Fractogel TSK HW-50 (S) (Salomonsson & Sundberg, 1994) gels by SEC or on Ultrahydrogel-120, -250 or -500 by high-performance liquid chromatography (HPLC) (Batey & Curtin, 1996). Amylose content is then calculated by comparing the population of very long chains (dp $>$ 100) attributed to amylose with that of shorter chains assumed to be generated from amylopectin. With native starches, accurate separation of amylose and amylopectin is not clearly evidenced with HPLC systems, and chromatographic yields are not always reported (Flamme, Jürgens & Jansen, 1994). HPLC has not provided good resolution between amylose and amylopectin, but excellent resolution has been obtained for some gels (e.g. Sepharose CL-2B) with low-pressure SEC (Colonna & Mercier, 1984; Wang, White, Pollack & Jane, 1993), although elution profiles are time-consuming and restrict the number of samples that can be analyzed.

A broad survey of the literature showed that values for amylose content depend on the method used, as already reported by some authors (Batey & Curtin, 1996; Sievert & Würsch, 1993). These methods all assume that amylose and amylopectin have very distinctive features and properties. Iodine-binding procedures disregard the slight reaction of amylopectin caused by structural differences relating to botanical origin, such as chain length or amount of branching. Moreover, the presence of intermediate fractions in starches does not always allow starch components to be clearly differentiated as amylose or amylopectin. Lansky, Kooi and Schoch (1949) found an "intermediate material" (IMI) characterized by a lower molecular weight than that of amylose and a slightly branched structure. These molecules showed intermediate responses, between amylose and amylopectin, to iodine-binding procedures and were mainly observed in amylomaize or wrinkled pea starches (Baba & Arai, 1984; Banks, Greenwood & Muir, 1974; Colonna & Mercier, 1984). Other authors referred to a similar "intermediate material" (IM2) to describe a third population of molecules observed in SEC profiles relative to intermediate elution volumes between those of amylopectin and amylose, particularly in the case of genetically modified maize starches (Kasemsuwan, Jane, Schnable & Robertson, 1995).

The purpose of this study was to determine which method should be used to measure amylose content accurately, regardless of the starch source (wild or mutant genotypes).

2. Experimental

2.1. Materials

Starches from various botanical origins were obtained from commercial sources: potato (Roquette Frères, France), smooth pea (Cosucra, Belgium), wrinkled pea (Amylose KG, Germany), wheat (Amylum, France) and normal maize (Roquette Frères, France). Genetically modified maize starches were a gift from Limagrain Genetics (France). Single- and double-mutant starches with various genetic backgrounds (W64A, F566, F546) were studied: *waxy* (*wx*), *dull* (*du*), *sugary*-2 (*su*2), *amylose extender* (*ae*), *ae wx*, *du su*2, *ae du* and *wx du*. For all methods used, the standard was potato amylose from Avebe (Foxhol, Netherlands).

L-α-Lysophosphatidylcholin (LPC, 99% pure) from egg yolk (Sigma), concanavalin A (Megazyme), amyloglucosidase (Merck), glucose–oxidase (Sigma) and peroxidase (Sigma) were commercial materials. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Size exclusion chromatography (SEC)

Forty milligrams of starch were dispersed in 2 ml of Me_2SO–H_2O, 90–10 (v/v) using gentle stirring for 2 days at room temperature (adapted from Delrue et al., 1992). Pure water (18 ml) was then added, and the solution obtained (2 mg per ml) was filtered on a 5-μm Millipore filter before being applied (5 ml) onto a column (2.6 × 200 cm) packed with TSK HW75 S gel (TosoHaas, Germany). The column was eluted with degassed pure water containing 0.02% NaN_3 at a flow rate of 10 ml per h. Void and total volumes of the chromatographic system were determined by the elution volume of the maximum concentration for amylopectin and glucose peaks, respectively. Fractions collected every 18 min were subjected to total carbohydrate content analysis by amyloglucosidase (procedure described below), and the maximum absorption wavelength of the iodine–carbohydrate complex was determined (see below).

Total carbohydrate content was measured before and after the filtering step by the orcinol–sulfuric acid method (Planchot, Colonna & Saulnier, 1997). Results were discarded if solubilization and filtration yields were not higher than 95%.

Chromatographic yields, determined from measurement of total carbohydrate content by the amyloglucosidase procedure on eluted fractions and injected solution, were above 90%.

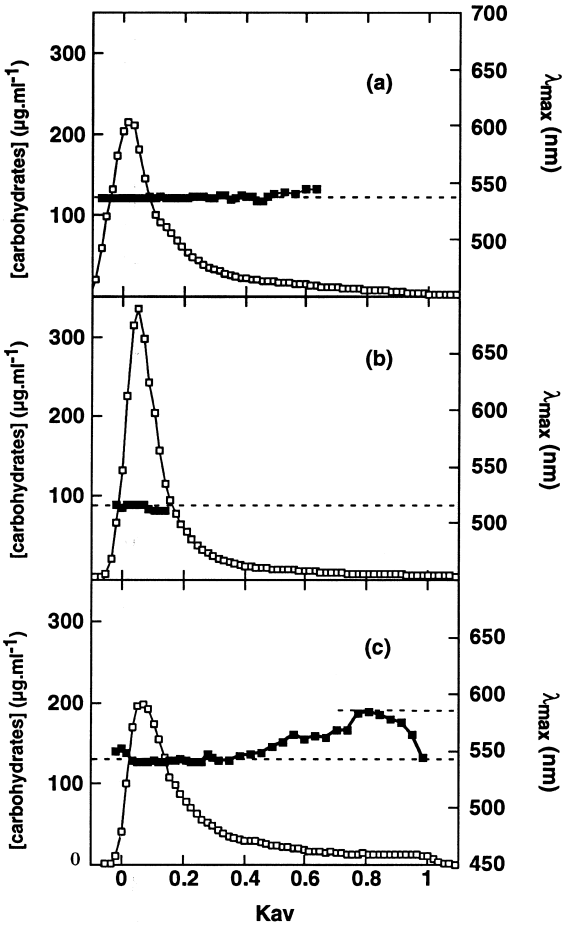

Fig. 1. SEC profiles of native starches on HW75 S. (a) *wx*, (b) *wxdu*, (c) *aewx*. –□– total carbohydrates (µg/ml). –■– λ_{max} (nm).

2.2.1.1. Measurement of carbohydrate content with amyloglucosidase. The pH of eluted fractions was adjusted to 4.7 with diluted acetic acid. Twenty microliters of amyloglucosidase solution (1 mg per ml) were added to 500 µl of eluted fraction, and starch macromolecules were then hydrolyzed at 60°C for 2 h under stirring. After cooling, the quantity of glucose produced was measured by the GOD–POD procedure (Planchot et al., 1997).

2.2.1.2. Measurement of the maximum absorption wavelength of the iodine–carbohydrate complex (λ_{max}). To 1 ml of eluted fraction were added 0.35 ml of 0.1 N KOH, 0.40 ml of 0.1 N HCl and 0.07 ml of I_2–KI solution (2% KI, 0.2% I_2), and λ_{max} was measured between 400 and 700 nm.

2.2.3. Iodine-binding capacity (IBC)

IBC was measured by the amperometric method (Larson et al., 1953). Starches were solubilized in 1 N KOH for 16 h at 4°C under magnetic stirring. To obtain an amylose concentration of around 1 mg per ml, starch concentrations were adjusted according to the presumed amylose content. Measurement was performed at 20°C after the linearity of IBC was checked according to amylose content (data not shown). The amylose content of the starch sample was deduced from the ratio of the IBC sample to that of pure amylose, i.e. 20.3 mg per 100 mg. Lipid extraction was done for wheat and maize starches according to the procedure of Morrison and Laignelet (1983), but slightly modified: starch was initially solubilized in Me_2SO–H_2O, 95–5 (v/v) for 3 days at room temperature under gentle stirring.

2.2.4. Calorimetric determination

Calorimetric determination of amylose content was done as proposed by Mestres et al. (1996). Differential scanning calorimetry (DSC) measurements were performed using a Setaram DSC 121 instrument. Sample weight was adjusted according to the presumed amylose content in order to obtain not more than 10 mg of amylose. Starch weights (w.b.) were between 10 and 20 mg. One hundred microliters of LPC solution (2% w/w in water) were added directly in the pan before hermetic sealing. A reference pan was filled with 100 µl of pure water and allowed to subtract the baseline. Samples were heated (3°C per min) from 25 to 165°C and cooled (−3°C per min) to 5°C. Energy data were collected during cooling, and the enthalpy of complex formation was determined. Amylose content was deduced from the ratio of sample enthalpy to that of pure amylose ($\Delta H \approx 25\ J\ g^{-1}$). This procedure was less time-consuming and gave equivalent results to those for a calibration curve (Sievert & Holm, 1993).

2.2.5. Concanavalin A method (Con A)

Amylose content was determined using a commercial assay kit from Megazyme (Ireland) according to the procedure described by Gibson et al. (1997). After dissolution of starch in acetate–salt solution (200 mM, pH 6.4), amylopectin was specifically precipitated by addition of Con A and removed by centrifugation (10,000 × *g*). Total carbohydrate contents in the initial solution and the supernatant were determined by (i) hydrolysis to glucose, using an amyloglucosidase, and (ii) colorimetric measurement of the resulting concentration by a glucose oxidase–peroxidase procedure, as previously described. Amylose content was deduced by the ratio of supernatant concentration to that of the initial solution. Minor modifications consisted in (i) initial starch dissolution in 2 ml of Me_2–SO–H_2O, 90–10 (v/v), (ii) defatting of starch samples, as for IBC measurements, and (iii) determination of glucose content by the GOD–POD procedure (Planchot et al., 1997).

3. Results and discussion

SEC shows the distribution of the hydrodynamic radius for all macromolecules in starch samples. In our study, starches were graded into three groups according to the number of peaks observed on their SEC profiles (Figs. 1–3). The first, second and third groups were composed of

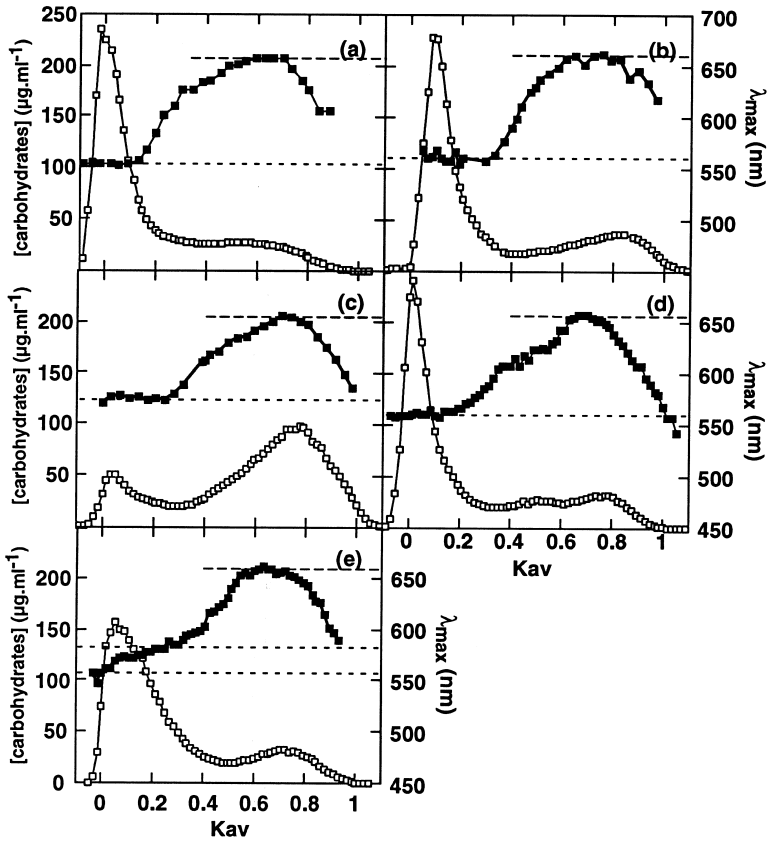

Fig. 2. SEC profiles of native starches on HW75 S. (a) potato, (b) smooth pea, (c) wrinkled pea, (d) normal maize, (e) wheat. –□– total carbohydrates (μg/ml). –■– λ_{max} (nm).

starches showing one, two and three peaks, respectively. In the elution profiles, the first peak ($K_{av} < 0.1$)—with a $\lambda_{max} < 600$ nm—corresponds to amylopectin, which was checked by applying a standard amylopectin sample. Amylopectin, because of its large hydrodynamic radius ($R_H \approx 200$ nm; Roger et al., 1999), was then excluded and eluted at void volume. The third peak ($0.7 < K_{av} < 0.8$) corresponds to amylose, which was checked by applying a standard amylose sample ($R_H \approx 17$–30 nm for $M_w \approx 0.3$–1.10^6 g mol^{-1}; Roger et al., 1996). The second peak ($0.4 < K_{av} < 0.5$) corresponds to a polysaccharide fraction showing an intermediate R_H value between that of amylopectin and amylose.

3.1. Starches showing a single-peak profile

According to the profile for total carbohydrate content (Fig. 1), *wx*, *wx du* and *ae wx* starches were included in a single population group. All fractions collected for these starches showed λ_{max} values lower than 600 nm and were composed only of amylopectin. Both *wx* and *wx du* amylopectins (Fig. 1a and b) showed constant λ_{max} values (535 and 520 nm, respectively) over the entire peak, and *wx du* amylopectin eluted as a narrow peak (0.35 K_{av} units) indicative of low polydispersity. The λ_{max} values ranged from 545 to 580 nm for *ae wx* amylopectin (Fig. 1c), indicating the presence of another component. However, amylopectin with a λ_{max} value at 545 nm represented up to 85% of *ae wx* starch.

The results for determination of the amylose content of *wx*, *wx du* and *ae wx* starches using SEC, IBC, DSC and ConA procedures are shown in Table 1.

Regardless of the method used, no amylose was detected in *wx* and *wx du* starches (<1%). The absence of amylose in *wx* or *wx du* starches has been reported by various authors (Gibson et al., 1997; Kugimiya & Donovan, 1981; Sievert & Holm, 1993; Wang et al., 1993; Yeh, Garwood & Shannon, 1981) who used either SEC on CL2B gel, amperometric titration of iodine affinity, DSC, or Con A.

However, the amylose content found in *ae wx* starch was

Table 1
Amylopectin λ_{max} (nm) and amylose content in starches from group 1. (SEC: size exclusion chromatography, Con A: concanavalin A, DSC: differential scanning calorimetry, IBC: iodine-binding capacity, nd: not detected; standard deviation in parentheses)

	Amylopectin λ_{max} (nm)	% Amylose			
		SEC	Con A	DSC	IBC
wx	535	nd	nd	nd	nd
wx du	520	nd	nd	nd	nd
ae wx	545–580	nd	7 (0.5)	nd	14 (2)

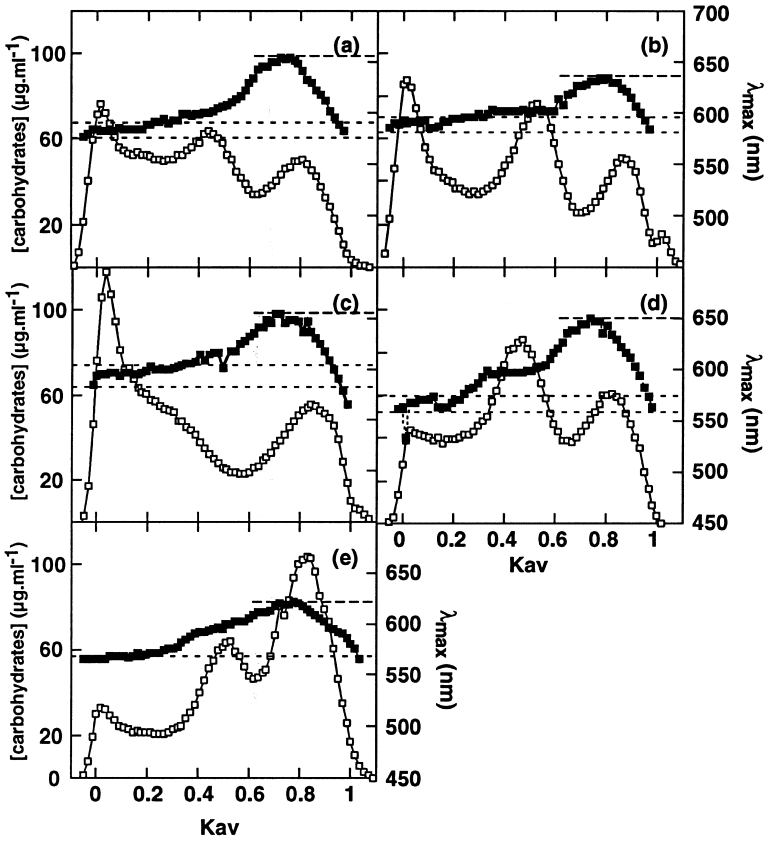

Fig. 3. SEC profiles of native starches on HW75 S. (a) *du*, (b) *su*2, (c) *dusu*2, (d) *aedu*, (e) *ae*. –□– total carbohydrates (µg/ml). –■– λ_{max} (nm).

dependent on the method used. DSC and SEC measurements were similar (amylose content <1%), whereas the IBC and Con A methods detected apparent amylose contents of 14 and 7%, respectively. These results are in agreement with those of Boyer, Garwood and Shannon (1976) and Bradbury and Bello (1993), who reported overestimated values of amylose content ($\approx$ 20%) in *ae wx* starch for iodine procedures. Apparently, branched *ae wx* amylopectin molecules contributed to the values obtained by the IBC method. This phenomenon was also observed with SEC, for which λ_{max} values were increased above K_{av} 0.5 either because of (i) slight contamination by amylose molecules or (ii) structural changes in amylopectin molecules of lower R_H. For SEC fractions with λ_{max} values of 580 nm (around K_{av} 0.8), amylose contamination was probably below 13%, as checked by estimation of the λ_{max} values of amylopectin and amylose mixtures at the same concentrations (data not shown). As these fractions represented less than 15% of total *ae wx* starch, amylose probably accounted for less than 2% (15×0.13) of this total. This value is too low for significant detection by IBC or DSC. Iodine response resulting from structural changes of some amylopectin molecules would indicate that linear regions of amylopectin were long enough to form an iodine complex with a λ_{max} value of 580 nm, i.e. at least 60 glucosyl units (John, Schmidt & Kneifel, 1983). However, such chain lengths would also have led to an overestimated value with DSC, as the minimum chain length for complex formation is below 30 (Helbert, 1994) with this method. This means that DSC is more sensitive than IBC to the presence of α-1,6 linkages.

The amylose content in *ae wx* starch as determined by the ConA method was 7%. As Con A reacts when the amount of non-reducing ends per molecule are sufficient, amylose and pullulan show no response, whereas amylopectin and glycogen respond since they are branched molecules with numerous branch points (Colonna et al., 1985). Moreover, an increase of external chain length induces an increase of the response with Con A, possibly because amylopectin could then form aggregates (Biton, 1984). As an increase of external chain length has been reported in *ae wx* as compared to *wx* amylopectin (Yun & Matheson, 1993), and as Con A was in excess amount in our conditions (4 mg per ml), the response of *ae wx* with Con A was greater than that of *wx*. Thus, the overestimation of *ae wx* amylose content cannot be explained by a difference in external chain length, which suggests that it may have been due to the presence of poorly branched low-molecular-weight amylopectin molecules. Bradbury and Bello (1993), who used high-performance SEC, also detected the presence of a low-molecular-weight amylopectin fraction in *ae wx* starch.

Our analyses of this first group of starches solely composed of amylopectin indicated that the fine structure

Table 2
Amylopectin λ_{max} (nm) and amylose content in starches from group 2 (standard deviation in parentheses)

	Amylopectin λ_{max} (nm)	% Amylose			
		SEC	Con A	DSC	IBC
Potato	555	23	24 (0.5)	26 (1)	21 (0.5)
Smooth pea	560	32	35 (1)	37 (2)	33 (0.5)
Wrinkled pea	570	69	71 (1)	70 (0.5)	65 (1)
Normal maize	560	26	24 (0.5)	34 (1)	27 (1)
Wheat	555–580	24	27 (2)	40 (0.5)	29 (1)

of this glucan was probably responsible for erroneous values in the determination of amylose content.

3.2. Starches showing a two-peak profile

The elution profiles for the second group, composed of potato, smooth pea, wrinkled pea, normal maize and wheat starches, are shown in Fig. 2a–e. The starches belonging to this group were composed of amylopectin and amylose, and these macromolecular fractions were eluted, respectively, before and after a threshold of K_{av} (around 0.4) when the variation of λ_{max} showed an inflexion point. For wrinkled pea starch, the SEC profile showed no resolution between amylose and an α-glucan fraction, IM1, previously reported by Colonna and Mercier (1984).

Potato, smooth pea, wrinkled pea and normal maize starches showed constant λ_{max} values for amylopectin (Fig. 2a–d, Table 2), which is significant for a homogeneous population. λ_{max} values of wheat amylopectin ranged from 560 to 580 nm (Fig. 2e), indicating that wheat amylopectin was composed of molecules with different structures. Banks and Greenwood (1975) reported 6–9% of amylopectin molecules in wheat starch, which showed an IBC of around 5 mg per 100 mg.

The results for determination of amylose content by the above-mentioned methods are shown in Table 2. Potato, smooth pea and wrinkled pea starches behaved simply, giving similar values for amylose content regardless of the method used, whereas the DSC method overestimated amylose content for normal maize and wheat starches.

In potato, smooth pea and wrinkled pea starches, amylose content was 23.5 ± 2, 35 ± 2.5 and 69 ± 3%, respectively, concordant with values reported with SEC (Flamme et al., 1994; Lloyd, Hedley, Bull & Ring, 1996), DSC (Kugimiya & Donovan, 1981; Sievert & Holm, 1993), and iodine-binding procedures (Colonna & Mercier, 1984; Larson et al., 1953; Morrison & Laignelet, 1983).

For maize and wheat starches, determination of amylose content showed, respectively, 25.5 ± 1.5 and 27 ± 2.5% as the mean values for SEC, ConA and IBC combined. These results are in agreement with those generally reported with SEC (Bradbury & Bello, 1993; Ikawa, Glover, Sugimoto & Fuwa, 1981), ConA (Gibson et al., 1997), or IBC (Boyer et al., 1976; Larson et al., 1953; Morrison & Laignelet, 1983). However, amylose content, as determined by DSC in our study, was higher for normal maize and wheat starches (37 and 40%, respectively). Mestres et al. (1996) reported an amylose content of 31.3% in normal maize starch with DSC. Using the same method, Kugimiya and Donovan (1981) found values of 33 and 37% in normal maize and wheat starch, respectively, whereas Sievert and Holm (1993) found 25 and 26%, respectively.

Overestimation with the DSC procedure suggests that some of the amylopectin linear chains were long enough to form complexes with the fatty acid of the lysolecithin, i.e. at least 30 glucosyl units, thereby increasing the enthalpy value for formation of amylose–lipid complexes. If this were the case, the IBC procedure should also have led to an overestimation of amylose content, which in fact, was not observed. Apparently, DSC overestimation did not result from the complexation of fatty acid with some amylopectin chains.

As Kugimiya and Donovan (1981), Mestres et al. (1996) and Sievert and Holm (1993) have shown that DSC experiments performed on defatted or non-defatted starches do not affect amylose determination, the overestimation reported for normal maize and wheat starches cannot be attributed to the presence of endogenous lipids. Kugimiya and Donovan (1981) reported that a low-molecular-weight amylose (LMW; 300 glucose units) with lysolecithin gave greater melting enthalpies than a high-molecular-weight amylose (HMW; molecular weight > 150,000). They indicated that this difference in enthalpies could result from (i) a difference in structure between HMW and LMW amyloses (perhaps due to a low degree of branching in HMW amylose) or (ii) the inability of some organized regions of HMW amylose to melt fully at experimental temperatures. With respect to the second hypothesis, Kugimiya and Donovan (1981) heated out only to 127°C during their DSC experiments, whereas Sievert and Holm (1993) found that a temperature below 170°C did not allow amylose to solubilize fully. As we heated out to 170°C, the second hypothesis cannot account for the overestimations observed. With respect to the first hypothesis of molecular weight variations in amylose, Klinger and Zimbalski (1992); Roger et al. (1996) showed that potato amylose had a higher molecular weight than maize or wheat amyloses. As our DSC experiments were performed with potato amylose as a standard, the first hypothesis could account for the above-mentioned overestimations. However, Sievert and Holm (1993) reported classical amylose content values for normal maize and wheat starches (25.4 and 26.1%, respectively), even though they used potato amylose as a standard. This parameter probably needs to be better controlled when using the DSC procedure.

In this second group of starches, even though the DSC procedure led to overestimated values for normal maize and wheat starches, quite similar results were observed for amylose content, regardless of the method used. This was due to the important structural differences between

Table 3
Amylopectin λ_{max} (nm) and amylose content in starches from group 3 (standard deviation in parentheses)

	Amylopectin λ_{max} (nm)	% Amylose			
		SEC	Con A	DSC	IBC
du	575–585	27	45 (0.5)	55 (1)	45 (4)
*su*2	580–590	24	50 (2)	58 (0.5)	45 (1)
*du su*2	580–605	34	58 (0.5)	66 (4)	60 (2)
ae du	560–570	30	56 (0.5)	64 (1)	56 (2)
ae	565	54	63 (1)	60 (2)	63 (3)

amylopectin and amylose, which made any of the available procedures suitable for analysis.

3.3. Starches showing a three-peak profile

The third group was composed of single and double maize mutant starches (*du*, *su*2, *du su*2, *ae du* and *ae*) for which the SEC elution profiles are given in Fig. 3a–e. The starches belonging to this group were composed of amylopectin and amylose—the two well-known starch macromolecules—and a third population eluting between amylopectin and amylose. This population, called intermediate material 2 (IM2), showed an R_H value between amylopectin and amylose. *dusu*2 starch belonged to the three-peak group since IM2 eluted as a shoulder on the amylopectin peak (Fig. 3c). All amylopectin λ_{max} values were quite high, which could have been due in part to an increase of amylopectin internal or external chain length as well as to IM2 contamination.

du, *su*2, *dusu*2 and *aedu* starches (Fig. 3a–d) showed non-constant λ_{max} values for amylopectin and λ_{max} values above 640 nm for amylose. Conversely, *ae* starch (Fig. 3e) showed constant λ_{max} values for amylopectin and λ_{max} values below 630 nm for amylose. The lower λ_{max} values reported for *ae* amylose were due to contamination by a high proportion of intermediate material IM1; as described by Banks and Greenwood (1975).

Many researchers have used SEC on CL 2B gel to characterize mutant starches of various botanical origins (Lloyd et al., 1996; Wang et al., 1993; Yeh et al., 1981). However, CL 2B gel cannot separate amylose from IM2 (Wang et al., 1993). For *du* starch, Wang et al. (1993) found amylose content values of 45.7 or 30.5% when SEC was used, respectively, with CL 2B on native starch or Bio-Gel P-6 on isoamylase debranched starch. These authors also observed differences between amylose content values when the above-mentioned SEC methods were used on native (76%) and debranched (57%) *aedu* starch, which confirms that IM2 cannot be detected by SEC with the CL 2B column used (2.6×79 cm). Their amylose content values were higher than ours, but difficult to compare because of differences in the genetic background of the *ae du* starches studied.

The results for the different methods of determining amylose content are shown in Table 3. For *du*, *su*2, *du su*2 and *ae du* starches, amylose contents were 18–34% higher with Con A, IBC or DSC methods as compared to SEC, but less than 10% higher for *ae* starch.

Regardless of the method used (Con A, IBC or DSC), amylose content was overevaluated compared to SEC values for *du*, *su*2, *ae du* and *du su*2 starches. For example, the amylose content in *du* starch was 27% when measured by SEC, whereas ConA and IBC gave a value of 45% and DSC 55%. SEC on HW75 S showed that the observed differences were due to the presence of IM2, whose reactivity to the different methods is unknown. However, regardless of the genotype considered among *du*, *su*2, *ae du* and *du su*2 starches, the highest overestimation was still obtained with the DSC method, as previously reported for normal maize and wheat starches.

The unexpected behavior of *ae* starch, as compared to other starches from the third group, might have been related to the presence of the intermediate material IM1, which elutes with amylose in SEC, accounting for the decreased amylose λ_{max} value (<630 nm). As *ae* starch is composed of four macromolecules (amylopectin, IM2, amylose and IM1), amylose content, as determined by SEC with HW75 S, was overestimated because of the impossibility of separating out IM1. This overestimation is concordant with the work of Kasemsuwan et al. (1995), who reported a lower amylose content in *ae* starch than in our study (33 vs. 54%), based on the difference in iodine affinity between whole starch and the amylopectin + Im2 mixture. However, these authors fractionated amylopectin and IM2 according to methods for which the reactivity of IM1 and IM2 is not quantitatively established.

The study of the third group of samples showed that determination of amylose content was difficult for genetically modified starches because of the presence of IM2. If SEC on HW75 S provides elution profiles with correct λ_{max} values for amylose, then it is the only suitable method. All the other tested methods resulted in overestimated values because of the poorly defined reactivity of IM2.

3.4. Structure of intermediate material 2

In maize, starch biosynthesis without some of the enzymes involved in granule construction resulted in the building up of a third macromolecule referred to as intermediate material 2 (IM2). Amylopectin and amylose were also present in the genetically modified starches, but IM2 was the most abundant population for *su*2 and *ae du* mutants (40 and 57%, respectively). The macromolecular structure of this polysaccharide is still unclear. It has been detected in the *sta*3 mutant of *Chlamydomonas reindhardtii* starch (Fontaine et al., 1993), the *ae* mutant of maize starch (Kasemsuwan et al., 1995) and the *su*2 mutant of maize starch (Takeda & Preiss, 1993). These mutants were all defective for a soluble starch synthase or a branching

enzyme. As different enzymatic defects are involved, IM2 could correspond to a family of different types of polymers rather than a single population.

According to SEC profiles on HW75 S, IM2 showed an intermediate hydrodynamic radius between amylopectin and amylose, with λ_{max} values of 600 nm, which is indicative of the presence of long chains between two α-1,6 linkages and would correspond to a dp of 100 (John et al., 1983). Thus, the resulting overestimation of amylose content with DSC and IBC methods would be due to the high reactivity of IM2 long chains with fatty acids and iodine. Moreover, the carbohydrate content determined with Con A did not correspond to the sum of amylose and IM2 contents, as would have been the case if IM2 were completely linear, indicating that a part of IM2 was sufficiently branched to precipitate with Con A. Thus, IM2 shows an intermediate size between amylose and amylopectin, and its macromolecular structure is characterized by branching points as well as long linear chains. Studies are in progress to investigate the influence of this unusual α-glucan on the structure of the starch granule.

4. Conclusion

The purpose of this study was to define the most suitable method for determining amylose content in starch. Analysis performed on normal and mutant starches from various botanical origins showed that SEC on HW 75 S gel led to accurate values, whereas CL 2B gel was not suitable for mutant starches. Measurement of IBC is still a standard method for comparing samples, though it can give incorrect values for mutant starches. DSC, which for unknown reasons overestimated values for maize and wheat starches, should be used with care. Response to Con A gave accurate values for normal starch, but erroneous values for mutant starches.

From a genetic point of view, this study showed that starch biosynthesis without some of the enzymes involved in granule construction produces a polysaccharide different from amylopectin and amylose. This polysaccharide shows an intermediate structure between the two well-known starch macromolecules, which creates difficulties for the amylose assay. Moreover, our experiments clearly indicate the structural heterogeneity of macromolecules between genotypes and within a specific genotype. The heterogeneity of amylopectin between genotypes is easily detected by different λ_{max} values, whereas the structural heterogeneity within a specific genotype is detected by non-constant λ_{max} values.

Even though low-pressure SEC (on an appropriate gel) is a time-consuming and repetitive method, it is still the only one that can provide numerous and accurate results concerning the macromolecular structure of starch. Thus, this method might be used more frequently, especially for the study of genetically modified starches.

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