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Determination of the amylose–amylopectin ratio of starches by iodine-affinity capillary electrophoresis

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Abstract

This paper describes the application of capillary electrophoresis to separate and quantify the main polysaccharide components, amylose and amylopectin, present in starch samples. The separation is based on the well-known affinity of these compounds to iodine. The starch components could be effectively separated in less than 7 min using an uncoated fused-silica 'bubble cell' capillary. The proposed method has been applied for the quantitative determination of the soluble amylose content and the ratio amylose–amylopectin in commercial starches. It is shown that the present method is reliable, gives detection limits in the order of 0.1 mg mL^{-1}, is faster than other methodologies reported in the literature, and can be easily applied to the analysis of different starches. In spite of differences in solubility of amylopectin from different sources, a reasonable estimate of the amylose–amylopectin ratio could be made. Additionally, it was shown that the resulting profiles obtained after hydrolysis with isoamylase and α-amylase can provide information on the structure of the starches studied.

Keywords: Starch; Affinity capillary electrophoresis; Complexation; Amylose; Amylopectin; Polysaccharides; Iodine

1. Introduction

Starch is one of the major polysaccharides used by plants for energy storage. It is widespread in seeds, roots and tubers as well as in stems, leaves, fruits and pollen [1]. Starches and their derivatives are versatile, non-toxic compounds, widely employed in numerous branches of industry, e.g., in food, paper, adhesive, textile and cosmetic applications [2,3]. Starch consists of extremely polydisperse anhydroglucose homopolymers in the form of amylose and amylopectin, in a proportion that depends on the botanical origin of the starch [4–6]. Native plant starches typically contain 20–30% amylose, but the amylose content may range from 0 to 80% [4]. Amylose is a linear or slightly branched polymer consisting of long chains with on average hundreds to thousands of glucose units connected with α-D-(1,4)-linkages (Fig. 1), with molecular masses of up to 2×10^6 [4,7,8]. Amylopectin, the major component of starch (70–80%), is a much larger, highly branched polymer consisting of relatively short segments of D-glucopyranose residues (20–25) linked by α-D-(1,4)-bonds, connected by α-D-(1,6)-glucosidic linkages (Fig. 1), with molecular masses ranging from 10×10^6 to 500×10^6 [4,8,9]. Genetically modified waxy maize, rice and potato starch consists solely of amylopectin, which has been found to be a favourable base material for chemically modified starches. Chemical modification can enhance certain starch properties, such as its solubility, which can be especially beneficial in non-food industrial applications [10].

Many of the physicochemical properties of starches that determine their suitability for particular end-uses are dependent on the ratio of amylose and amylopectin and on their molecular weight distributions [11–15]. In particular, the ratio of the two types of polysaccharides influences properties such as solubility, viscosity, gel formation and gelatinization (solubilization) temperature [12–15]. Therefore, the amylose content and amylose–amylopectin ratio of starches are important quality parameters in starch processing.

The amylose content and the amylose–amylopectin ratio in cereal starches have traditionally been measured

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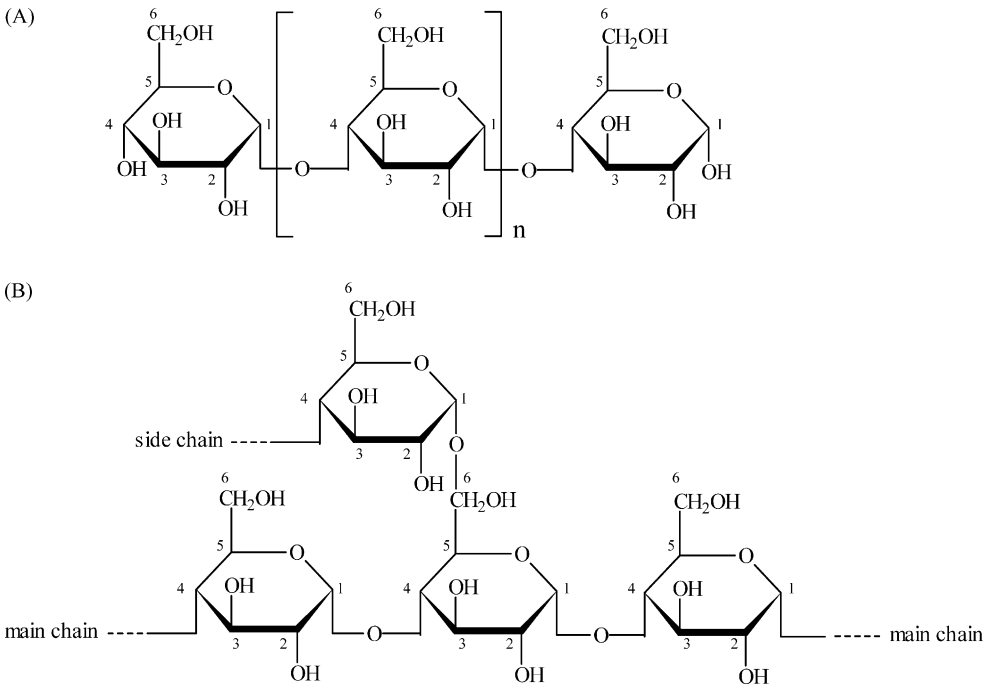

Fig. 1. Structures of amylose (A) and amylopectin (B) showing the two different types of chain linkages.

by iodine-binding procedures, whether amperometric [16], potentiometric [17], or spectrophotometric [18]. These procedures are based on the capacity of amylose to form helical inclusion complexes with iodine, which exhibit a blue colour characterized by a maximum absorption wavelength (λ_{max}) above 620 nm. However, these methods are subject to certain types of bias. Amylopectin also forms complexes with iodine, which reduces the concentration of free iodine measured by the non-colorimetric methods. The amylopectin–iodine complexes absorb at similar wavelengths ($\lambda_{max} \approx 540$ nm) and therefore interfere also with the measurement of amylose–iodine complexes in the colorimetric methods [17,19]. These problems lead to an overestimation of the amylose content, and correction procedures have to be applied. Although different approaches have been proposed [20–23], with none of these the problems could be overcome completely.

Alternative procedures for the measurement of amylose content have been developed. Gibson et al. [24] reported the selective precipitation of amylopectin with concanavalin A, which allows to determine the amylose content by measuring the amount of carbohydrates (glucose) in the supernatant. However, the mechanism of this precipitation has not been completely clarified, and this procedure is tedious and more complex than the amylose–iodine method.

Size-exclusion chromatography (SEC), either of native or debranched starch [25–27], has also been used to measure the amylose content. With isoamylase-debranched starch, the amylose content is calculated by comparing the population of very long chains [degree of polymerization (DP) > 100] attributed to amylose with that of shorter chains assumed to be generated from amylopectin. Although these approaches are useful for, e.g., fine-structure studies of the compounds, they are not very suited for large sample numbers. With native starches, an accurate separation of amylose and amylopectin is not always clearly shown with HPLC systems [28–31], and chromatographic yields are not always reported [32]. Excellent resolution between both components has been obtained by low-pressure SEC [33,34] with specific gels (e.g., Sepharose CL-2B), at the expense of long chromatographic run times. Therefore, fast and effective polysaccharide characterization techniques to assess the quality of starches are still required.

It has been previously demonstrated that capillary electrophoresis (CE) exhibits an extremely high separation efficiency in the separation of polysaccharide mixtures [35–40]. Borate buffers [39,41] have been employed to provide charge to the neutral sugar molecules by complexation. In addition, derivatization with UV absorbing [42], or preferably fluorescent [35,37,38,40] labels has been required for their detection. However, separation based on borate complexation appears to be limited to a maximum molecular mass of ca. 30 000. Higher oligosaccharides acquire a constant mass-to-charge ratio and, therefore, are not resolved each from other [43]. On the other hand, the derivatization (labeling) procedures are lengthy (4–24 h), and the detection of larger polysaccharides is problematic due to an incomplete labeling reaction (poor labeling efficiency at very high DP), leading to a loss of quantitative information and the possibility of heterogeneity in the derivatized products.

An alternative strategy, developed by Brewster and Fishman [44], to impart charge and to permit absorbance detection of polysaccharides, is through their characteristic complexation with iodine/triiodide (polyiodides). This so-called

iodine-affinity CE, has been employed for the separation of starch components [44,45] and other carbohydrates [46]. Although Brewster and Fishman [44] and Soini and Novotny [45] developed a procedure for classification of various starch-derived materials, no quantitative data were reported.

We report here the use of iodine-affinity CE to characterize starches from different botanical sources. Further, we have studied the resulting profiles obtained during hydrolysis of several starches with two different enzymes (isoamylase and α-amylase). The method was also applied for the quantitative determination of the soluble amylose content and the ratio amylose–amylopectin in commercial starches.

2. Experimental

2.1. Instrumentation

Uncoated fused-silica capillaries including a standard (37.0 cm × 75 μm i.d. × 375 μm o.d.) and an extended path-length capillary (37.0 cm × 75 μm i.d. × 375 μm o.d., bubble factor: 3) were obtained from Composite Metal Services (Ilkey, UK) and Agilent Technologies (Waldbronn, Germany), respectively. The CE experiments were carried out with a Beckman P/ACE 2100 instrument equipped with a diode array spectrophotometric detector.

2.2. Reagents and samples

Corn and potato amylopectin (Ap), potato amylose (Am), corn, waxy corn, rice and wheat starches were obtained from Sigma (St. Louis, MO, USA). Tapioca, waxy rice, modified waxy corn and rice starches were a gift of Dr. Hans-Gerd Janssen (Unilever Research and Development, Vlaardingen, The Netherlands). Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa*, and bacterial α-amylase (EC 3.2.1.1) were obtained from Sigma. Dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and other analytical-grade reagents as well as deionized water were used.

2.3. Sample preparation

Stock solutions of polysaccharides were prepared by magnetic stirring of dispersions containing 5–10 mg mL^{-1} of the analyte in 90% DMSO at room temperature until dissolution was complete. For quantitation studies, the sample solutions had to be heated; the vials were placed in an oven at 100 °C during 60 min.

2.4. CE procedure

Before first use, a new capillary was conditioned by flushing with 1 M NaOH, 0.1 M NaOH and water for 10 min each, and finally for 30 min with the running buffer. Between runs, the capillary was flushed with running buffer for 5 min. At the end of a working session, the capillary was rinsed with 0.1 M NaOH for 5 min, water for 5 min and N_2 for 1 min. The optimum running buffer was 20 mM acetic acid/sodium acetate, pH 5.0, with 1.2 mM I_2 and 7.2 mM KI. Standards or samples were injected hydrodynamically for 2 s at 3400 Pa (0.5 psi). Separations were performed at 25 °C, and the applied voltage was 20 kV. Detection of iodine complexes was at 560 nm, although the entire spectra in the range 300–600 nm were recorded.

2.5. Enzymatic treatment

For enzymatic debranching with isoamylase, the isoamylase enzyme solution was diluted with 50 mM acetic acid/sodium acetate, pH 3.7 (25 μL solution in 2 mL 50 mM acetic acid/acetate buffer, pH 3.7). To an aliquot (0.2 mL) of solubilized starch solution isoamylase (50 μL ∼ 780 units) was added, and the debranching reaction was carried out at 37 °C in a water bath for 24 h. After incubation with the enzyme, the digested starch solution was heated at 100 °C for 20 min to inactivate the enzyme, centrifuged at 14 000 × *g* for 15 min, and the supernatant was injected into CE system.

For the enzymatic hydrolysis with α-amylase, starch samples were hydrolyzed by the action of α-amylase according to the method described by Franco et al. [47] with some modifications. An aliquot (0.2 mL) of starch solution was mixed with acetic acid/acetate buffer, pH 5.2 (0.8 mL) and was thermostated at 20 °C for 15 min in a water bath. After taking a 0.1 mL sample, 2 units (∼5 μL) of α-amylase were added, and again samples (0.1 mL) were taken after 1, 5, 20, 40, 60, and 120 min of incubation. The samples were immediately boiled for 10 min, centrifuged at 14 000 × *g* for 15 min, and injected into the CE system.

3. Results and discussion

3.1. Optimization studies

The initial CE conditions used for the analysis of amylose (Am) and amylopectin (Ap) were based on the previous methodologies reported in the literature [44,45], and modified to allow an accurate quantitation of both polysaccharides in starch materials. Hong et al. [46] studied the complexation between maltodextrin oligomers (with DP < 50) and polyiodides by CE. In this study, the authors showed the importance of a 'critical oligomer length' in the complexation process, and its dependence on experimental conditions, especially on the iodine–iodide (I_2/I^-) ratio present in the background electrolyte (BGE). Thus, we investigated the influence of this ratio on the separation of starch components. Fig. 2A shows the variation of the electrophoretic mobility of Am and Ap with iodine (I_2) concentrations at several I_2/I^- molar ratios.

The I_2 concentration was varied from 0.2 to 2 mM. At lower I_2 concentration, the Ap peak was not completely resolved from the electroosmotic flow (EOF) peak. At higher I_2 concentrations (>2 mM), broad peaks were observed, and

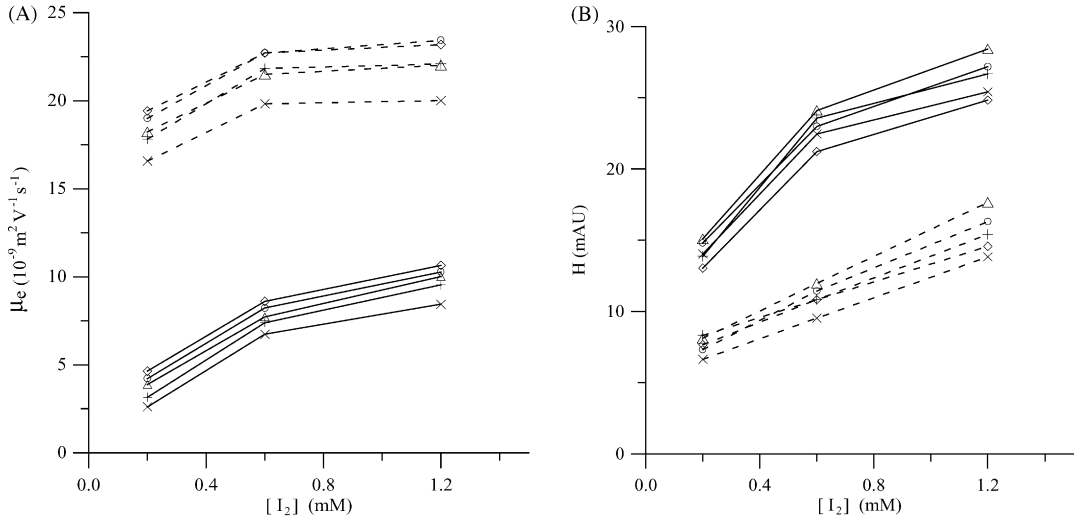

Fig. 2. Effect of I_2 concentration on the electrophoretic mobilities (A) and peak height (B) of Ap (—) and Am (- - -) at different I_2/I^- molar ratios: (◊) 1/12, (◯) 1/6, (+) 1/2, (△) 1/3 and (×) 2/3. BGE: 20 mM acetic acid/acetate at pH 5; capillary: 37 cm (effective length 30 cm) × 75 μm i.d.; applied voltage: 20 kV; hydrodynamic injection: 0.5 psi for 2 s; detection at 560 nm.

the migration time repeatability was poor. The I_2/I^- ratio was varied from 0.08 to 0.65. Limits to the value of this ratio were set by the solubility of iodine on the one hand and problems with Joule heating on the other. As can be seen in Fig. 2A, the mobility of Am is higher than that of Ap in a I_2/I^- containing BGE under all conditions tested. The mobility of Ap was found to increase steadily with the iodine concentration, while that of Am reached a maximum at a relatively low I_2 concentration (<0.6 mM). This shows that the long linear polyglucose chains of Am form stronger polyiodides complexes than the relatively short branches of Ap. These results are consistent with those reported in literature [44,45,48], and reflect the much shorter average chain length of Ap in comparison with Am.

However, the effect of the I_2/I^- ratio on the electrophoretic mobilities was relatively small; a slight increase of the mobilities was observed with decreasing ratio (increasing iodide concentration). Consequently, differences in the oligomer chain length (in the complexation process) dependent on the I_2/I^- ratio could not be established due to high DP of these compounds, in contrast with the findings found by Hong et al. [46] with short oligosaccharides (DP < 50).

Further, the influence of iodine concentration on detection sensitivity was studied by measuring the peak height of each analyte at different I_2 concentrations and I_2/I^- molar ratios (Fig. 2B). The sensitivity obtained for both polysaccharides is dependent on the I_2 concentration, while non-significant changes are observed between different I_2/I^- ratios.

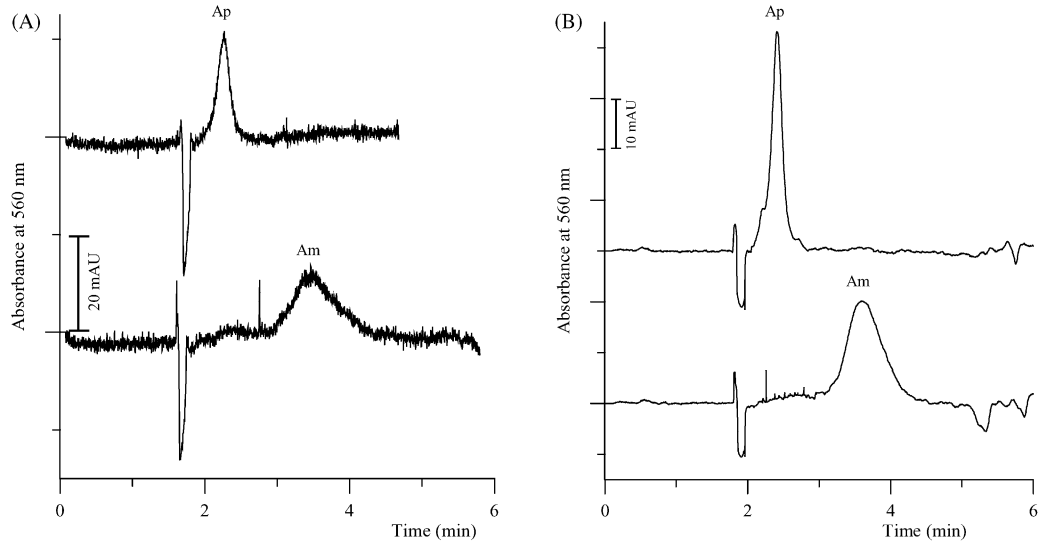

Fig. 3. Electropherograms of Ap and Am from potato using a standard 75 μm i.d. capillary (A), and a 75 μm i.d. 'bubble cell' capillary (B); bubble factor: 3. BGE: 20 mM acetic acid/acetate at pH 5, with 1.2 mM I_2 and 7.2 mM KI. Other conditions as in Fig. 2.

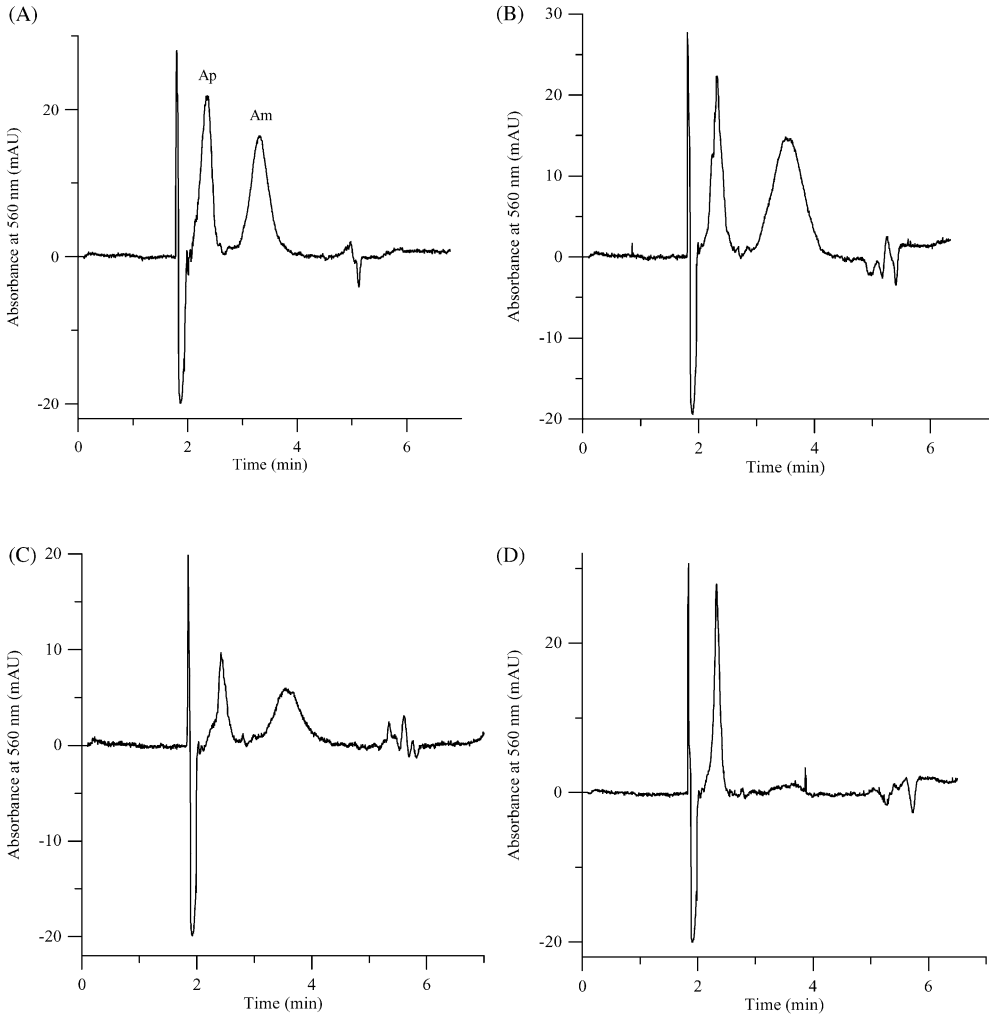

Fig. 4. Electropherograms of (A) corn, (B) wheat, (C) tapioca and (D) waxy rice starches. Separation conditions as in Fig. 3.

As a result from this study, a BGE was chosen containing 1.2 mM I_2 and 7.2 mM KI, which gave the best compromise between resolution and sensitivity for both analytes. Under these conditions (Fig. 3, left part), Am and Ap are well resolved from each other, in a short time, with Am migrating as a broad peak. This electropherogram could be favourably compared to other procedures reported in literature [44,45].

The iodine-binding capacity of Am and Ap and the maximum absorption of their complexes is known to depend on the oligomer chain length of polysaccharides [49,50]. At a wavelength of 560 nm, both Am–iodine and Ap–iodine complexes could be detected [44,45], and also the quantitation is relatively independent of small variations in the oligosaccharide composition [44–46]. However, at this wavelength the sensitivity of detection is not high enough to perform an accurate quantitation of the analytes and to obtain the corresponding Am–Ap ratio. Thus, in order to improve the method sensitivity, a capillary with extended light path ('bubble cell') was used (Fig. 3, right part). A significant improvement (a factor of ∼2–3 was obtained for both analytes) of the signal-to-noise ratio is clearly shown, while the resolution of both peaks remained practically unchanged with the bubble cell. Fig. 4 shows the electrophoretic profiles of several types of starches, where differences in Am–Ap ratio were easily distinguishable according to the different botanical sources (Table 2).

3.2. Enzymatic studies

To study the mechanism of the iodine-affinity separations, enzymatic studies with isoamylase and α-amylase have been performed. Isoamylase digestion has been used to determine the Am–Ap ratio of starches [25–27]. Isoamylase completely debranches the Ap molecule, and the digestion products of regular starches comprise a high-DP fraction (>500) from Am, and a low-DP fraction consisting of the short chains of debranched Ap. The two fractions are then separated by SEC and quantified to find the Am–Ap ratio of the original starch.

A waxy corn starch consisting of pure Ap was treated with isoamylase. Fig. 5 shows the electropherograms obtained with this sample before (trace A) and after (trace B) enzymatic digestion. In the electropherogram of debranched

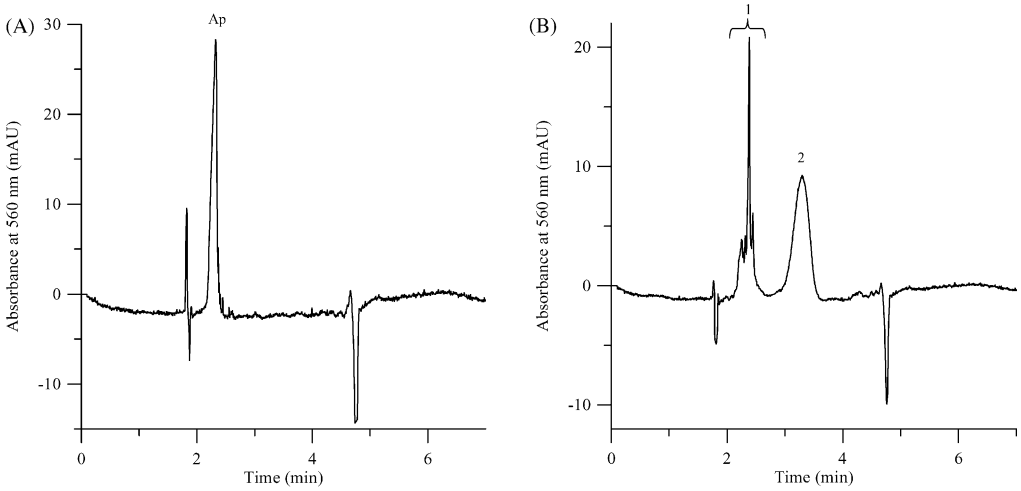

Fig. 5. Electropherograms of a waxy corn sample before (A) and after (B) debranching with isoamylase. Peak identification: (1 and 2) enzymatic degradation products, for details see text. Separation conditions as in Fig. 3.

Ap, a set of low-mobility peaks is present, in the time-region of the original Ap peak, and a wide peak close to the typical migration time of Am. Sargeant [25] and Batey and Curtin [27] have reported that the debranching of Ap yields two fractions of linear chains: A-chains with a DP in the order of 15–20, and B-chains with a higher DP (∼45).

In our experiments, these A-chains could be responsible for the peaks in the 2–3 min range ('fingerprint-region'), while the B-chains elute as the broad high-mobility peak. Apparently, the mobility of the polyiodide complexes of these longer B-chains is similar to that of Am–iodine complexes. Interestingly, while the peak pattern of the fingerprint-region was similar for all starch digestions tested, the relative intensity of the peak ensemble differed between starches. This opens up the possibility to study branching structures of Ap samples with this method.

Debranching was also performed with Am samples (data not shown). The electropherograms of the resulting products showed some small peaks in the fingerprint-region, which confirms the presence of minor amounts of α-(1,6)-branches in the Am structure [4,7,8].

A similar study was performed with α-amylase. Fig. 6 shows the electropherograms obtained during the digestion of an Am sample. As can be seen in the figure, α-amylase hydrolyzes the α-D-(1,4)-linkages of Am in a random manner [51,52]. During the digestion of Am, at first, peaks become visible in the fingerprint-region from the short oligosaccharides chains formed during enzymatic breakdown. Later, these peaks disappear again when the oligosaccharides are completely broken down to glucose or another saccharides [53,54]. The glucose chains (the resulting oligosaccharides) formed during the initial stage of the digestion by α-amylase are apparently long enough to form polyiodide complexes [46,51]. When Ap samples were treated with α-amylase, the short-chain peaks in the first part of the electropherograms could not be observed. This might be partly caused by overlap with the original Ap peak. However, it may also indicate that the oligosaccharides formed from Ap are too short to form polyiodide complexes [46,51].

3.3. Quantitation of starch samples

Under the proposed conditions, several starch samples were analyzed. However, the presence of a large number of spikes in the electropherograms, especially with cereal starches, hinders an accurate quantitation of starch components. Cereal starches (corn, rice, etc.) do not solubilize readily at room temperature as does potato starch. Therefore,

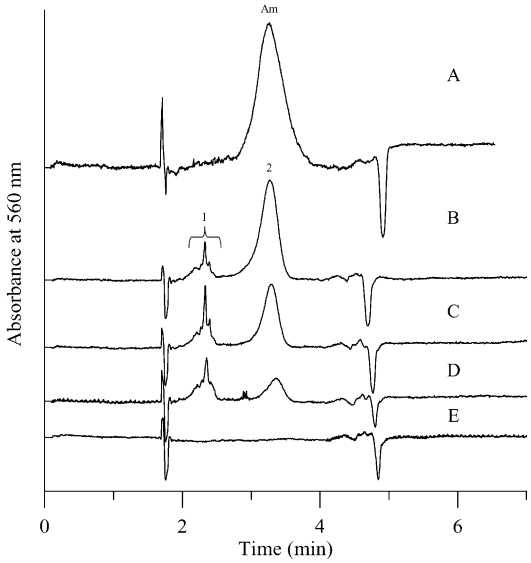

Fig. 6. Electropherograms of Am from potato without (A) and with α-amylase at incubation times of 5 min (B), 10 min (C), 20 min (D) and 60 (E) min. Peak identification: (1 and 2) enzymatic degradation products, for details see text. Separation conditions as in Fig. 3.

Table 1
Repeatability and limit of detection (LOD) for S/N = 3 of amylopectin (Ap) and amylose (Am) in the proposed procedure

Analyte	LOD (mg mL^{-1})	t_m (R.S.D., %)		Area (R.S.D., %)	
		Intra-day	Inter-day	Intra-day	Inter-day
Ap corn	0.13	0.2	0.6	2.3	2.1
Ap potato	0.05	0.3	0.5	2.6	2.8
Am potato	0.2	1.4	2.1	3.1	4.6

Table 2
Determination of the Am–Ap ratio in several starches

Starch samples (manufacturer)	Reference		Found (%)	
	Am (%)	Ap (%)	Am[a]	Ap[a]
] Am reference (Megazyme)	70	0	68.8 ± 1.2	ND[b]
Corn (Sigma)	27	73	26.5 ± 0.7	72.7 ± 1.8
Waxy corn (Sigma)	0	100	ND[b]	100
Rice (Sigma)	–	–	21.2 ± 0.9	79.1 ± 1.6
Wheat (Sigma)	–	–	28.8 ± 1.4	71.6 ± 1.2
Tapioca (Avebe)	–	–	19.7 ± 1.1	81.1 ± 1.9
Waxy rice (Remy Industries)	–	–	ND[b]	100

[a] Means (on dry basis) and standard deviation of triplicate analysis.
[b] Not detected.

we decided to dissolve the starches at elevated temperature. With long heat treatment (12–24 h at 100 °C), the solubility of starch granules increases [55], but a degradation of some starches occurs. The latter is especially significant for potato starch, which shows an extensive depolymerization (breakdown) [18,32,56–58].

Morrison and Laignelet [18] and Grant et al. [31] have reported that most starch material can be readily dissolved in 60–90 min at 100 °C. When this procedure was applied, the presence of spikes was decreased significantly. This sample treatment was chosen as a compromise between complete solubilization, and the possibility to be applied as routine method for the estimation of Am and Ap.

Calibration curves were established with six standard solutions of each solute (Am from potato and Ap from corn and potato) up to 5 mg mL^{-1}. Straight lines with $r > 0.998$ were obtained. The detection limits were in the order of 0.1 mg mL^{-1}, and peak area and migration time repeatabilities were satisfactory (see Table 1). The proposed method was applied for the determination of the Am–Ap ratio in several commercial starch samples (Table 2). However, notable differences in the content of Ap were found depending on the type of Ap used for calibration. This can be explained by the different solubilization of Ap from different sources. Apparently, the solubility of Ap from corn is more comparable to that of other starches (wheat, rice, tapioca) than Ap from potato [18,56]. With the proper calibration standards, a good agreement was observed between the declared composition and the concentrations found.

Several samples of modified starches were also analyzed. In all cases, only very small peaks were observed in the electropherograms. Modification of starches usually involves their substitution with sulphate, phosphate or acetate groups. The presence of such negatively charged groups will diminish the iodine (I_3^-) affinity of the starches, and with that the absorption [59]. A comparison of the UV–vis spectra of native and modified starches (data not shown) in the presence of iodine confirmed this hypothesis.

4. Conclusions

It has been shown that iodine-affinity CE can be used effectively to separate and quantify the principal polysaccharides components in starch samples from different botanical origins. The present procedure gives lower detection limits and is faster than other protocols reported in the literature. It can be easily applied to the routine quality control of starches. Further, our protocol does not require long derivatization steps and coated capillaries, thus substantially reducing analysis costs. In spite of differences in solubility of amylopectin from different sources, a reasonable estimate of the amylopectin content, and the amylose–amylopectin ratio could be obtained for different commercial starch samples.

Additionally, the enzymatic approaches described here can give information about the molecular structure of the starch components, constituting a promising tool in the study of starch and its derivatives.

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