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High-performance anion-exchange chromatography–electrospray mass spectrometry for investigation of the substituent distribution in hydroxypropylated potato amylopectin starch

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Abstract

The use of high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) coupled on-line with electrospray mass spectrometry (ESI-MS) for analysis of the substitution pattern in chemically modified starch, has been investigated. In order to characterise the distribution of substitution groups along the polymer chain, hydroxypropylated potato amylopectin starch (HPPAP) was subjected to enzymic hydrolysis, followed by analysis of the degradation products by HPAEC-PAD-MS. When using conventional chromatographic techniques for characterisation of enzymic hydrolysates, standard compounds are required for identification of the hydrolysis products. However, the on-line coupling with ESI-MS allowed identification of all products obtained, substituted as well as unsubstituted, and also of those compounds that co-eluted, without the need for standards. Further, HPAEC-PAD-MS was shown to be useful for analysis of the substitution pattern in modified starch; from results obtained it was suggested that the hydroxypropyl groups were homogeneously distributed in the amylopectin molecule. It was also shown that the starch hydrolysing enzymes were hindered by the hydroxypropyl groups and preferentially cleaved glucosidic linkages between unsubstituted glucose units.

Keywords: Substituent distribution; Enzymic degradation; Hydroxypropylated amylopectin

1. Introduction

Starch is often chemically modified by the introduction of different functional groups in order to obtain products with desired properties for certain applications. One important product group is hydroxypropylated potato starch that has a wide range of industrial applications, e.g., as thickener in food and food-related products or as coating material in paper products [1]. It is well known that functional properties of the final starch product depend on type and amount of substituent, but also on the distribution of substituents in the polymer [2,3]. In order to direct the modification process towards a certain product with desired properties, it is necessary to have knowledge about the relationships that exist between the modification process, functional properties, and the substitution pattern. As the correlations between these parameters are not fully understood, considerable efforts are being made in the development of analytical methods for structural

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characterisation of modified starches [4–8]. Several methods have been reported for determination of the molar substitution (M.S., average number of moles of substituent per glucose unit) [9,10], degree of substitution (D.S., average number of hydroxyl groups substituted per glucose unit) [11,12], and the substitution pattern on a monomer level [9,13,14]. However, the distribution of substituents along the polymer chain has shown to be more difficult to determine, and up to date relatively few papers concerning methods for analysis of the substitution pattern on a polymer level have been published [5,7,8,15,16]. One approach is to partially degrade the modified starch with enzymes having different selectivities. The enzyme action is hindered by the substituents [17–20], thus modified starch is hydrolysed to a lesser extent compared with unmodified starch. Subsequent characterisation of the hydrolysis products by different analytical methods can give information about the homo- and heterogeneity of the distribution of substituents along the polymer chain. Analysis of oligosaccharides in enzymic hydrolysates has commonly been performed by means of size-exclusion chromatography (SEC) [4,7], high-performance anion-exchange chromatography (HPAEC) [21,22], or by determination of the reducing power and/or glucose content in the hydrolysate [4,8,15]. However, although liquid chromatographic techniques enable good separation and detection of carbohydrate hydrolysates, especially HPAEC with pulsed electrochemical detection (PAD) [23,24], it is not possible to identify the hydrolysis products obtained without access to reference material. The lack of standards is an obvious problem when investigating enzymic hydrolysates of chemically modified starches, as these hydrolysates very likely contain numerous different substituted oligosaccharides, of which there are no commercial standards available. A relatively new hyphenation of techniques that has proved to be very useful in the analysis of oligosaccharides is HPAEC coupled on-line with mass spectrometry (MS) [25,26]. The combination of HPAEC with MS detection is very convenient in analyses where the elution order of different compounds is unpredictable, analytes co-elute, or reference compounds are unavailable [27,28]. In structural studies on chemically modified starch, mass spectrometry should be a very appropriate technique, one reason being that substituted oligomers differ from unsubstituted ones by their mass rather than their conformation.

We herein report a method where HPAEC-PAD coupled on-line with electrospray mass spectrometry (ESI-MS) was applied in the characterisation of chemically substituted products in enzymic hydrolysates of hydroxypropylated potato amylopectin starch. The aim of the study was to investigate the potential of this combination of techniques in analyses of the substituent distribution in chemically modified polysaccharides. Hydroxypropylated amylopectin samples were degraded by α-amylase and amyloglucosidase, and subsequent determination of the hydrolysis products by HPAEC-PAD-MS made it possible to draw conclusions about the substitution pattern. Additionally, it was demonstrated that information about the hydrolytic action of enzymes on modified polysaccharides could be achieved by means of HPAEC-PAD-MS.

2. Experimental

2.1. Chemicals

Potato amylopectin starch (PAP, amylose deficient potato starch) and hydroxypropylated PAP modified in granular slurry (HPPAPg, M.S.=D.S.=0.16 [8]) or polymer solution (HPPAPs, M.S.=D.S.=0.14 [8]), were gifts from Lyckeby Stärkelsen (Kristianstad, Sweden). HPPAPg was prepared in a heterogeneous reaction where native PAP was mixed with water and Na_2SO_4. Propylene oxide was added and the modification reaction proceeded under alkaline conditions at 40°C for 48 h. The mixture was neutralised before washing and drying the product. Preparation of HPPAPs was carried out in a homogeneous reaction where native PAP was dissolved in water at 95°C for 1 h. The remaining modification procedure was carried on as described above. Glucose, fructose, maltose, maltotriose, maltotetraose, and maltopentaose (degree of polymerisation, DP 1–5) were from Sigma Chemicals Co. (St. Louis, MO, USA). α-Amylase (EC 3.2.1.1) from *Aspergillus oryzae* (cat. No. E-BARBP) and amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* (cat. No. E-AMGPU) were obtained from Megazyme Interna-

tional (Bray, County Wicklow, Ireland). Chromatographic eluents were prepared from 50% w/w NaOH (J.T. Baker, Deventer, Netherlands) and NaOAc (Merck, Darmstadt, Germany). The water used in all experiments was purified in a Milli-Q system, Millipore (Bedford, MA, USA).

2.2. Enzymic degradation

PAP (10 mg) or HPPAP (20 mg) samples were dissolved in water (4 ml) at 100°C for 30 min. The pH was adjusted to 5.0 with 0.4 *M* citrate buffer and the solution was incubated with α-amylase (30 U) at 40°C for 20 h. Subsequently, the pH was adjusted to 4.0 with 0.4 *M* citrate buffer, amyloglucosidase (30 U) was added, and the starch solution was further hydrolysed at 40°C for another 20 h. After deactivation of the enzymes by boiling at 100°C for 10 min, the solution was ultrafiltrated in Vivaspin Concentrators (Lot no. 99vs0239, Vivascience, Binbrook Hill, Binbrook Lincoln, UK) having a membrane with a molecular mass cut-off (MWCO) of 10 kDa, for removal of the enzymes. Before injection onto the chromatographic system the solution was diluted 1:10.

2.3. Chromatographic system

Analysis of the hydrolysis products was carried out using HPAEC-PAD coupled to MS. The chromatographic system (Dionex 500; Dionex, Sunnyvale, CA, USA) was controlled by PeakNet™ software (Dionex) and consisted of a GP40 gradient pump, a Rheodyne injector (Cotati, CA, USA) equipped with a 20 μl injection loop, a CarboPac PA-100 guard- and analytical column, and an ED40 electrochemical detector. The detector had the following waveform: E_1=0.10 V (t_d=0.20 s, t_1=0.20 s), E_2=0.70 V (t_2=0.19 s), and E_3=−0.75 V (t_3=0.39 s) vs. a $Ag/AgCl_{(sat)}$ reference electrode and a gold working electrode. Separation was performed at a flow-rate of 0.3 ml/min using a gradient programme with 300 m*M* NaOH (A) and water (B) as eluents. Between 0 and 30 min, eluent A increased linearly from 15 to 95%, and was then held constant at 95% between 30 and 110 min. Between every chromatographic run the column was regenerated with 500 m*M* NaOAc prepared in 150 m*M* NaOH (with the mass spectrometer disconnected). The waste outlet of the electrochemical detector cell was connected to the MS interface via an anion self-regenerating suppressor (P/N 46081, ASRS-II, Dionex), that acted as a desalting device for removal of the Na^+ ions from the eluent to neutralise the OH^- ions prior introduction to MS [28]. Desalting was accomplished by electrolysis of water at 500 mA by means of a Dionex SRS Controller. The water reservoir was pressurised by nitrogen resulting in a water flow-rate of approximately 3 ml/min. The H_3O^+ ions produced at the anode were exchanged for Na^+ ions by two cation-exchange membranes. The H_3O^+ ions combined with OH^- ions from the eluent to form water, whereas the Na^+ ions combined with OH^- ions produced from the cathode to form NaOH that was collected as waste.

2.4. Mass spectrometry

The desalted eluent was directed to the electrospray ionisation (ESI) interface of an Esquire-LC mass spectrometer from Bruker Daltonik (GmbH, Bremen, Germany), equipped with an ion trap mass analyser. The mass spectrometer was operated at the following voltages: $V_{endplate}$=3500 V, V_{cap}=4000 V, $V_{cap\,exit}$=100 V, $V_{skimmer1}$=35 V, and $V_{skimmer2}$=8 V. Nitrogen was used as nebuliser gas at a pressure of 30 p.s.i. and acted also as drying gas at a flow-rate of 7 l/min with the temperature kept at 350°C. The gas was supplied to the mass spectrometer by a nitrogen generator from Whatman (Haverhill, MA, USA). The spectra were acquired in the positive ion mode with multiple ion detection (MID), scanning from m/z (mass-to-charge ratio) 150–1500.

3. Results and discussion

When investigating the distribution of substituents in modified polysaccharides by means of enzymic hydrolysis, different analytical methods are required in order to identify the hydrolysis products. HPAEC-PAD is a very suitable technique for analysis of saccharides in enzymic hydrolysates [29–31], but the use of this method alone is limited in several aspects. However, the combination with MS enables determination of saccharides both when analytes co-elute

and the elution order is unknown, but above all MS allows identification of peaks without the need for sample standards. These superior characteristics of MS were employed as a complement to HPAEC-PAD for the determination of chemically substituted products in enzymic hydrolysates of hydroxypropylated starch.

3.1. HPAEC-PAD

Native potato amylopectin starch (PAP) and hydroxypropylated PAP (HPPAP) were subjected to extensive hydrolysis by α-amylase; an endo-enzyme that randomly cleaves α-D-(1→4)-glucosidic linkages in polysaccharides, yielding oligosaccharide degradation products, and amyloglucosidase; an exo-enzyme that starts from the non-reducing end and catalyses the successive hydrolysis of terminal α-D-(1→4)- and (1→6)-linkages with the release of monomeric glucose in its β-form [32]. Unmodified potato starch should mainly be degraded to glucose but also insignificant amounts of phosphodextrins [24], as potato amylopectin contains phosphate groups (one per 200–300 glucose units [33,34]) that the enzymes cannot by-pass. However, hydrolysis of hydroxypropylated starch could result in release of, aside from glucose, a broad range of substituted mono- and oligomers, due to the fact that the enzymes are hindered by the hydroxypropyl groups [8]. In order to characterise the hydrolysis products obtained, the hydrolysates were analysed by HPAEC-PAD.

Separation of mono- and oligosaccharides on the anion-exchange resin in the chromatographic column depends on their pK_a values. Substitution of the free hydroxyl groups in the anhydroglucose units (AGU) leads to changes in pK_a, changes that depend on type and position of the substitution group. Hydroxypropylation of a saccharide should increase the pK_a and thereby decreasing the retention time, as the hydroxyl group in the hydroxypropyl group is less acidic compared with the hydroxyl group in the unsubstituted AGU. This is due to the alkyl group in the hydroxypropyl group, which rejects electrons and therefore decreases the degree of ionisation of the hydroxyl group. In addition, hydroxypropylation at C-2 should lead to a lower pK_a than hydroxypropylation at C-6, as the former position is more acidic due to its proximity to the electron withdrawing acetal group at the anomeric centre [35]. These slight differences in acidity could under optimal conditions result in selective interactions with the anion-exchange resin, thus leading to different retention times.

Fig. 1a shows the chromatogram from HPAEC-PAD analysis of the hydrolysate of unmodified PAP.

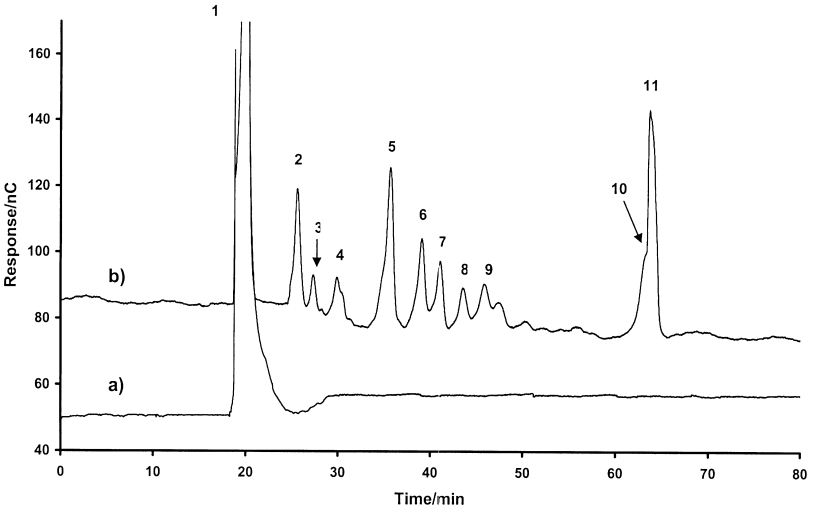

Fig. 1. (a) HPAEC-PAD chromatogram from analysis of α-amylase and amyloglucosidase hydrolysate of PAP. (b) HPAEC-PAD chromatogram from analysis of α-amylase and amyloglucosidase hydrolysate of HPPAPg.

As expected, the only hydrolysis product that was possible to identify was glucose (determined by spiking with standard), although the glucose peak had a shoulder that indicated the presence of additional products. The analysis of HPPAP hydrolysates resulted in several additional peaks (Fig. 1b). The first peak was determined as glucose, but identification of the remaining peaks was not possible. The lack of specific information in this chromatogram clearly demonstrates the limitations using HPAEC-PAD alone in those cases where no standard substances are available. Most certainly, the unidentified peaks corresponded to substituted products, and in order to investigate which, the HPPAP hydrolysates were further subjected to HPAEC-PAD-MS analysis.

3.2. HPAEC-PAD-MS

The main problem when coupling HPAEC-PAD to MS is the solvent conditions used in the chromatographic separation, which are incompatible with the MS detection. The high ion concentration in the mobile phase (i.e., 0.3 *M* NaOH) causes severe salt deposits in the ion source, which significantly hampers the MS analysis. Yet another problem is the formation of Na^+ ion clusters that cause a high chemical background noise. However, avoiding high NaOH concentrations results in limitations of the maximum degree of polymerisation (DP) that can be eluted from the column and thereby determined by HPAEC-MS. To circumvent this problem, a cation-exchange membrane-desalting device that efficiently exchanges Na^+ ions in the eluent for H_3O^+ ions, was introduced between the chromatographic system and the MS interface [28,36].

Sugar oligomers readily form cationic adducts with alkali metal ions, e.g., Na^+ or Li^+ [37], therefore the mass spectra of the hydrolysis products were acquired in the positive ion mode. As the mobile phase used in the chromatographic separation contained a high concentration of Na^+ ions, the analytes were present as strongly sodiated molecules, despite desalting the eluent before MS analysis. This is exemplified in Fig. 2, where maltose substituted with one hydroxypropyl group is shown in its monosodiated form (m/z=423). Previous work has shown that sugars carry approximately one charge per 1000 Da, although for oligosaccharides only singly ($[M+Na]^+$) and doubly ($[(M+2\ Na)/2]^{2+}$) sodiated molecules have been observed so far [38].

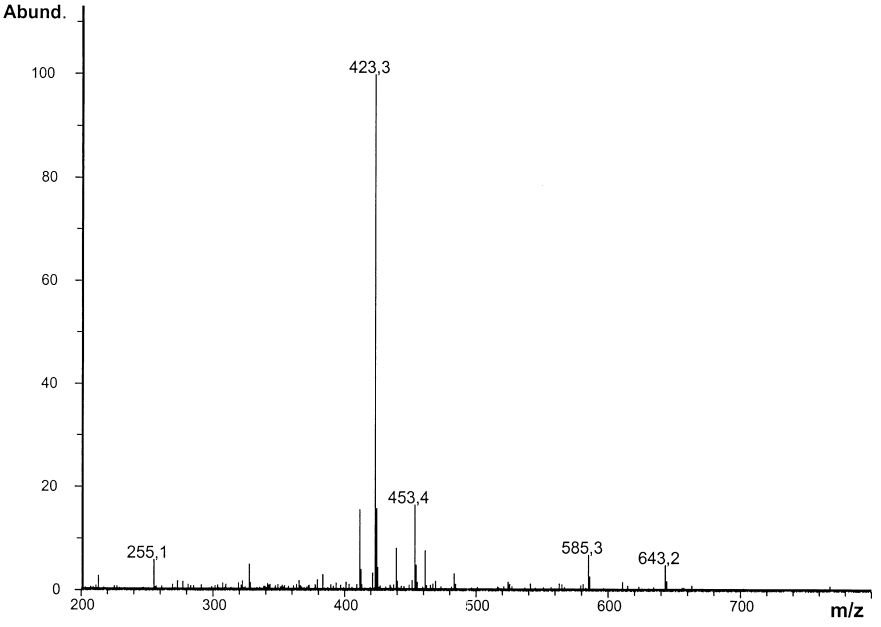

Fig. 2. Mass spectrum of monosodiated *O*-(2-hydroxypropyl)-maltose with m/z=423 obtained from α-amylase and amyloglucosidase hydrolysis of HPPAPg.

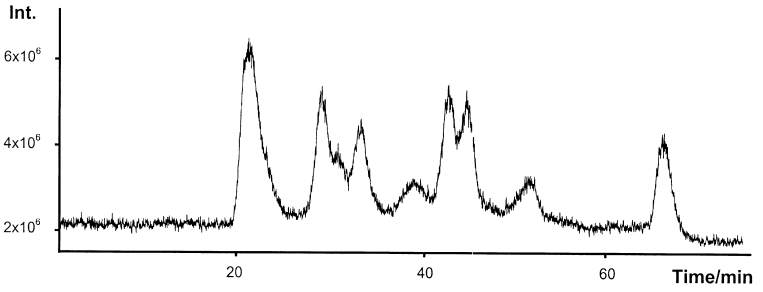

Fig. 3. TIC chromatogram obtained from HPAEC–ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of HPPAPg.

The total ion current (TIC) chromatogram obtained from HPAEC-PAD-MS analysis of the HPPAP hydrolysate, which is shown in Fig. 3, resembles the chromatogram obtained from the HPAEC-PAD analysis (Fig. 1b), except a band broadening and shift in retention time owing to the dead volume in the desalting device between the outlet of the electrochemical detector and the MS interface. In order to identify the peaks, mass chromatograms for expected mass-to-charge ratios of all possible hydrolysis products were constructed and searched for the presence of peaks. This could be a quite tedious procedure, as the theoretical number of possible substituted mono- and oligomers is large. However, herein, this number was limited because the M.S. and the distribution of hydroxypropyl groups in the AGU in the HPPAP samples were known from a previous investigation by Richardson et al. [8]. In that study it was shown that the presence of disubstitution in the AGU was very low and that trisubstitution did not exist at all, therefore it was assumed that no hydrolysis products substituted with more than two hydroxypropyl groups per glucose unit were present. This assumption reduced the number of products that had to be searched for.

Table 1 summarises the components found in the enzymic hydrolysates of HPPAP when analysed by HPAEC-PAD-MS. All mono- and oligomers were present as monosodiated molecules; disodiated products were not found at all. The mass chromatogram in Fig. 4 shows the presence of glucose ($m/z=203$), a result that confirmed what was already known from the HPAEC-PAD analysis alone. However, in the HPAEC-PAD chromatogram (Fig. 1b) the major peak has a retention time equivalent to that of pure glucose, whereas in the mass chromatogram two separated peaks with $m/z=203$ appeared. The first of these two peaks with the same mass-to-charge ratio corresponds to glucose, whereas the identity of the second peak is unknown. The presence of this extra peak with the same mass-to-charge ratio as glucose is not understood. Surprisingly, this peak has the same retention time as fructose (which also has $m/z=203$), although a presence of fructose in these hydrolysates cannot be explained. However, the result accounts for the peak shoulder in Fig. 1a that shows the products obtained from enzymic hydrolysis of unmodified PAP; this peak is with MS detection shown to consist of two compounds with the same mass-to-charge ratio; glucose and an unknown compound. One minor peak was observed at $m/z=261$ (Fig. 5), which corresponds to glucose substituted with one hydroxypropyl group ($\Delta M_{\text{hydroxypropyl}}=58$). Monosubstitution of a glucose unit can result in three different monomers; glucose substituted at C-2, C-3, or C-6, respectively, but the mass chromatogram for $m/z=261$ reveals only one

Table 1
m/z ratios of the products found in the hydrolysates

DP[a]	n[b]	$[M+Na]^+$	Peak no. in HPAEC-PAD chromatogram
1	0	203	1
1	1	261	–
2	1	423	1, 4
2	2	481	1
3	1	585	2, 3, 5, 6, 7
3	2	643	5
4	1	747	8, 9, 10
4	2	805	11
5	2	967	–

[a] Degree of polymerisation.
[b] Number of hydroxypropyl groups.

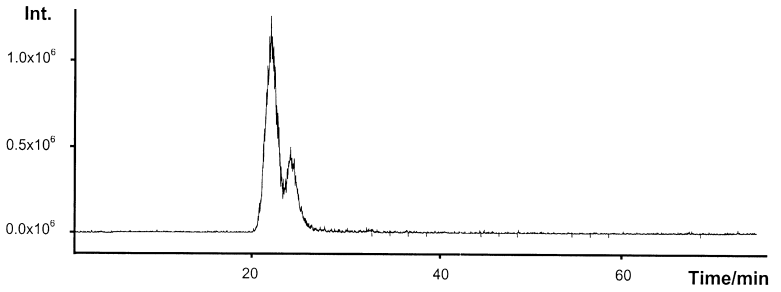

Fig. 4. Mass chromatogram for $m/z=203$ from HPAEC–ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of HPPAPg.

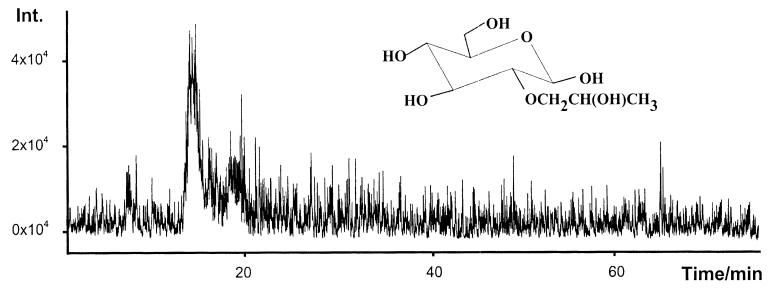

Fig. 5. Mass chromatogram for $m/z=261$ from HPAEC–ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of HPPAPg.

distinct peak. It is known that the molar ratio of substitution at C-2, C-3, and C-6 in the AGU is of about 12, 1, and 1%, respectively [8], therefore it is highly probable that this peak corresponds to 2-*O*-(2-hydroxypropyl)-glucose. It is also possible that the chromatographic conditions do not allow separation of 2-*O*-, 3-*O*-, and 6-*O*-(2-hydroxypropyl)-glucose, i.e., they co-elute and the single observed peak contains all three compounds. Monosubstituted glucose cannot be seen in the HPAEC-PAD chromatogram, a fact that demonstrates the need for MS detection as a complement to PAD. Fig. 6 shows the mass chromatogram for $m/z=423$, which corresponds to maltose substituted with one hydroxypropyl group. The appearance of two well separated peaks with $m/z=423$ having approximately the same intensities can probably be explained by two isomeric forms of *O*-(2-hydroxypropyl)-maltose. As hydrolysis of modified starch with α-amylase in combination with amyloglucosidase previously has shown to mainly produce oligomers with at least one unsubstituted glucose unit at the reducing end [39], these two isomers should bear the hydroxypropyl group on the non-reducing moiety of the maltose

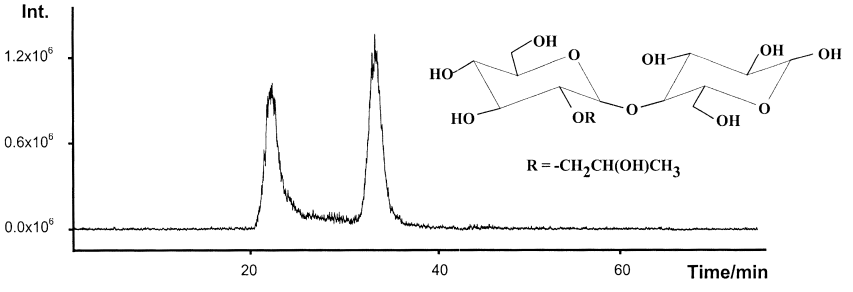

Fig. 6. Mass chromatogram for $m/z=423$ from HPAEC–ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of HPPAPg.

unit. Further, considering the relative substitution of the AGU (see above) it is unlikely that the separation is caused by different positions of the hydroxypropyl group in the AGU; the majority of the substituents has previously been shown to be located at C-2 [8]. However, one possibility is that the two separated peaks correspond to the diastereoisomers of *O*-(2-hydroxypropyl)-maltose, i.e., C-2 in the hydroxypropyl residue is in the R and S configuration in a ratio of approximately 1:1. Maltotriose and maltotetraose substituted with one hydroxypropyl group ($m/z=585$ and $m/z=747$, respectively) each gave rise to five and three peaks, respectively, in their mass chromatograms. In accordance with the above discussion, it was suggested that the appearance of several separated peaks with the same mass-to-charge ratio is due to oligomers substituted with a single hydroxypropyl group at different positions in the oligomer and/or diastereoisomers of the oligomers. In order to elucidate the exact position of the hydroxypropyl groups in the oligomers, i.e., on which of the glucose units in the oligomer the substituents were located, it is necessary to label the reducing ends and further investigate the hydrolysates with sequential MS^n analysis. This type of structural analysis can successfully be performed on an ion trap mass spectrometer, whereas triple quadropole systems are impossible to use in sequential MS–MS analysis. However, this was judged to be outside the scope of this initial study and is the topic of a continued work. Further, the hydrolysates were found to contain small amounts of disubstituted maltose ($m/z=481$), maltotriose ($m/z=643$), and maltopentaose ($m/z=967$), whereas disubstituted maltotetraose ($m/z=805$) was present in significantly higher amounts. Although no absolute quantitative data of the individual components were possible to obtain, due to the lack of standards, a rough estimation of the proportions of each product was made from the intensities in the mass chromatograms.

According to the results obtained, no oligomers with DP higher than five were detected by HPAEC-PAD-MS (Table 1). To make sure no components in the hydrolysate remained in the column, experiments with the MS disconnected and gradients with high NaOAc concentrations were performed. NaOAc is required for elution of higher oligomers, but was not used in combination with the MS as the presence of acetate ion clusters caused a high chemical background signal. In this case, the use of gradients with high NaOAc concentrations (200–500 m*M*) did not reveal any additional peaks in the HPAEC-PAD chromatogram, compared with analysis where the mobile phase contained no acetate. Therefore, it was assumed that all components eluted from the column without the need for acetate.

3.3. Substituent distribution

According to the results obtained from the HPAEC-PAD-MS analysis, mainly glucose and short, substituted oligomers (DP 2–5) are present in the hydrolysates of the HPPAP samples. This suggests that hydroxypropyl groups are relatively homogeneously distributed in the amylopectin chains. If the distribution of hydroxypropyl groups is statistically random, the main substituted hydrolysis products should be monosubstituted glucose, maltose, and/or maltotriose, depending on whether the enzymes cleave linkages next to substituted units or not. This statement can be explained by considering the molar substitution of the two HPPAP samples (M.S.=0.14 and 0.16 for HPPAPs and HPPAPg, respectively). Upon α-amylase and amyloglucosidase hydrolysis, these samples should, in addition to unsubstituted glucose, be degraded to monosubstituted glucose, maltose, and/or maltotriose. Although the HPPAP hydrolysates contain relatively low amounts of disubstituted maltotriose, maltotetraose, and maltopentaose units, the main hydrolysis products are monosubstituted maltose, maltotriose, and maltotetraose. This implies that the distribution of hydroxypropyl groups resembles a statistically random distribution. A more blockwise or heterogeneous distribution of substituents would have resulted in the release of longer oligomers with a higher degree of substitution in addition to, or instead of, the short, low substituted oligomers, that were released in this case. In this particular case, where the M.S. of the samples is low, it cannot be expected to get highly substituted oligomers even for a more heterogeneous distribution pattern, unless there is an extreme clustering of hydroxypropyl groups.

The type and amount of hydrolysis products obtained from the two HPPAP samples prepared under different conditions were similar, indicating

that the choice of modification procedure (granular slurry or solution) did not affect the homogeneity of the substitution to any greater extent.

3.4. Enzyme action

When studying the products obtained from α-amylase and amyloglucosidase hydrolysis of HPPAP determined by HPAEC-PAD-MS (Table 1), it could be concluded that α-amylase together with amyloglucosidase were unable to degrade the polymer completely, due to the hydroxypropyl groups that hindered the enzyme action. The occurrence of high amounts of monosubstituted compared with di- and trisubstituted maltotriose and maltotetraose, suggests that both α-amylase and amyloglucosidase preferentially cleave glucosidic linkages between unsubstituted glucose units in the polymer. This is supported by the fact that monosubstituted glucose, disubstituted maltose, di- and trisubstituted maltotriose, tri- and tetrasubstituted maltotetraose, and tetra- and pentasubstituted maltopentaose were present in very small amounts or not found at all in the hydrolysates. Similar results on enzymic digestibility of hydroxypropylated starch have been reported previously by several authors [40–43]. However, the detection of small amounts of monosubstituted glucose suggests that α-amylase and/or amyloglucosidase occasionally do cleave glucosidic linkages next to hydroxypropylated glucose units. Further, the presence of monosubstituted maltose and disubstituted maltotriose supports the suggestion that one, or both, of the enzymes can tolerate linkages next to substituted units, although it is not possible to determine whether it is both or just one of the enzymes that possess this ability without labelling the reducing end. In an investigation on methylated starch by Mischnick [44] it was suggested that amyloglucosidase has the ability to cleave linkages adjacent to substituted glucose units, whereas α-amylase accepts glucosidic linkages between unsubstituted and substituted glucose units only if the substituents are located at certain positions in the AGU. It has also been demonstrated that the activity of hydrolases on glucosidic linkages adjacent to substituted units depends on the nature, number, and position of substituents [21,45].

3.5. Comparison of PAD versus MS

The results obtained in this investigation illustrate the convenience of using simultaneously PAD and MS detection when analysing chemically modified oligosaccharides. The two techniques are based on different detection principles; PAD is an electrochemical detection technique that relies on the electrochemical conversion of the analyte, whereas MS detection depends on the ionisation capacity, i.e., formation of Na^+-complexes. Consequently, the detectors produce two different responses for one single analyte, and these responses have shown to serve as good complements to each other.

The main advantage with MS detection is the possibility to construct separate mass chromatograms for every analyte. This leads to a significant reduction of the chemical noise level, and detection of analytes that cannot be seen in the PAD chromatogram becomes possible. This is demonstrated in Figs. 1b and 5, where the peak corresponding to monosubstituted glucose ($m/z=261$) is not seen in the PAD chromatogram (Fig. 1b), whereas it is readily detected in the mass chromatogram for $m/z=261$ (Fig. 5). Additionally, analytes that co-elute give rise to one single peak in the PAD chromatogram and cannot be detected individually, whereas the individual mass chromatograms for every analyte allow separate detection of those having the same retention time. As an example, glucose (Fig. 4) and monosubstituted maltose (Fig. 6) co-elute, both corresponding to the first peak in the PAD chromatogram. Without MS detection with individual mass chromatograms, it would have been impossible to reveal the presence of the two analytes having the same retention time, as there are no commercial standards of hydroxypropylated maltose available. As a consequence, the first peak could easily, but erroneously, have been assigned to glucose only. Furthermore, MS more easily detects sugars that are difficult to oxidise. On the other hand, for sugars that are difficult to ionise, PAD could be the better detector choice. In quantitative analysis, both the PAD and MS detector have several drawbacks. The relative response factor for the PAD detector varies with DP [30,46], type of substituent, and number of substituents [21]. Therefore, an absolute calibration for each compound to be quantified is required, which in turn requires access

to standards of these compounds. The MS response also varies with different analytes and in addition, the response for an analyte is very sensitive to variations in the instrument settings, such as voltages, temperatures, and gas flows.

4. Conclusions

An analytical methodology has been presented, where the substituent distribution in, and the enzymic action on, hydroxypropylated potato amylopectin starch (HPPAP) were investigated by means of HPAEC-PAD in combination with ESI-MS. HPAEC coupled to ESI-MS was shown to be a very useful combination of techniques for characterisation of substituted products obtained from α-amylase and amyloglucosidase hydrolysis of HPPAP. When using PAD alone, it was impossible to identify the substituted oligomers, as there are no standards available of these compounds. However, the combination with MS detection allowed identification of the substituted hydrolysis products without the need for standards, as well as of those analytes that co-eluted. Identification of the components in the hydrolysates made it possible to study how the hydroxypropyl groups affected the enzyme action; α-amylase and amyloglucosidase cleaved preferentially glucosidic linkages between unsubstituted AGUs, although the presence of monosubstituted glucose and maltose, and disubstituted maltotriose units indicated that hydrolysis of linkages adjacent to substituted AGUs did occur. In addition, a detailed characterisation of the hydrolysate content gave some information about the distribution of substituents along the polymer chain. Mainly short, low substituted oligomers were detected in the hydrolysate of the two HPPAP samples, suggesting a relatively random distribution of hydroxypropyl groups along the amylopectin chains.

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