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Enzyme-Aided Investigation of the Substituent Distribution in Cationic Potato Amylopectin Starch

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The distribution of substituents along the polymer chain in cationic potato amylopectin starch, modified in solution, granular slurry, or dry state, was investigated. The starch derivatives were successively hydrolyzed by different enzymes, followed by characterization of the hydrolysis products obtained by means of electrospray mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). ESI-MS and MALDI-MS were proved to be appropriate techniques for identification of the substituted hydrolysis products, for which there are no standard compounds available. No highly substituted oligomers were found in the hydrolysates, which was taken as an indication of a more or less homogeneous distribution of cationic groups in the amylopectin molecules. Furthermore, from the results obtained it was suggested that the enzymes cleave glucosidic linkages only between unsubstituted glucose units and, preferentially, linkages in sequences containing more than two adjacent unsubstituted units. The determination of the amount of unsubstituted glucose produced from every successive hydrolysis step revealed slight differences between the different starch samples with respect to the homogeneity of the substitution pattern. Among the three samples under investigation, starch cationized in solution was found to have the most and dry-cationized starch the least homogeneous distribution of substituents.

Modified starches are produced in large quantities for use in a broad range of industrial areas. The main purpose of the modification is to achieve desired changes in the physical and chemical properties of the starch product, to improve the functionality in certain applications. However, the properties of modified starches depend on several factors, such as modification reaction, type and degree of substitution, and distribution of substituents. Thus, to efficiently manufacture a certain starch product, knowledge of the correlations between the modification reaction, the substitution pattern, and the final properties is required. As a consequence, it becomes important to characterize the substituent distribution, both on a monomer and on a polymer level. The substitution pattern on a monomer level, that is, the distribution of substituents in the anhydroglucose unit (AGU), has been determined for a large number of starch derivatives, usually by nuclear magnetic resonance spectroscopy[1,2] or gas chromatography/mass spectrometry.[3,4] However, less is known about the distribution of substituents inside the starch granule, in the amorphous and crystalline regions, and along the starch chains. One approach to investigate the distribution on a polymer level is to hydrolyze the starch with selective enzymes, followed by characterization of the hydrolysis products by different analytical techniques, for example, size-exclusion chromatography,[5,6] high-performance anion-exchange chromatography (HPAEC),[7] or MS.[8,9] The main advantage of an enzymic hydrolysis is that the enzyme action is hindered by the substituents;[10−12] thus, a selective hydrolysis of the starch is achieved. Comparison of the products obtained from enzymic hydrolysis of modified starch with those from native, unmodified starch can provide information on the location of the substituents in the polymer. In previous studies on various modified starches based on an enzymic approach, it has been suggested that substitution occurs preferentially in amylose and in the regions around the branching points in amylopectin, which constitute the amorphous areas in the semicrystalline granule.[5,6,10,13,1413−14]

Cationic starches are widely used in paper manufacturing as wet-end additives, surface sizes, and binders of the cellulose

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(1) Xu, A.; Seib, P. A. *J. Cereal Sci.* **1997**, *25*, 17−26.
(2) Heins, D.; Kulicke, W.-M. *Starch/Stärke* **1998**, *50*, 431−437.
(3) Wilke, O.; Mischnick, P. *Starch/Stärke* **1997**, *49*, 453−458.
(4) Richardson, S.; Nilsson, G. S.; Bergquist, K.-E.; Gorton, L.; Mischnick, P. *Carbohydr. Res.* **2000**, *328*, 365−373.
(5) Steeneken, P.; Woortman, A. *Carbohydr. Res.* **1994**, *258*, 207−221.
(6) Kavitha, R.; BeMiller, J. N. *Carbohydr. Polym.* **1998**, *37*, 115−121.
(7) Manelius, R.; Buléon, A.; Nurmi, K.; Bertoft, E. *Carbohydr. Res.* **2000**, *329*, 621−633.
(8) Steeneken, P. A. M.; Tas, A. C.; Woortman, A. J. J.; Sanders, P. In *Starch: Structure and Functionality*; Frazier, P. J., Richmond, P., Donald, A. M., Eds.; Special Publication; The Royal Society of Chemistry: Cambridge, 1997; Vol. 205, pp 153−162.
(9) Richardson, S.; Cohen, A.; Gorton, L. *J. Chromatogr., A* **2001**, *917*, 113−123.
(10) Hood, L. F.; Mercier, C. *Carbohydr. Res.* **1978**, *61*, 53−66.
(11) Östergård, K.; Björck, I.; Gunnarsson, A. *Starch/Stärke* **1988**, *40*, 58−66.
(12) Mischnick, P. *Starch/Stärke* **2001**, *53*, 110−120.
(13) van der Burgt, Y. E. M.; Bergsma, J.; Bleeker, I. P.; Mijland, P. J. H. C.; van der Kerk-van Hoof, A.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1998**, *312*, 201−208.
(14) Manelius, R.; Nurmi, K.; Bertoft, E. *Cereal Chem.* **2000**, *77*, 345−353.

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fibers.[15] The most common commercial cationic starch is prepared by chemical reaction of the free hydroxyl groups in the AGUs of the starch molecule with a quaternary ammonium reagent. The modification reaction has traditionally been carried out under alkaline conditions in a granular suspension of starch.[15,16] More recently, an alternative dry cationization reaction has been developed, in which the modification reagent is mixed with dry starch powder.[17,18] In addition, a homogeneous reaction can be employed, in which the starch is completely dissolved in water before adding the modification reagent. Cationization of the single AGU exhibits high preference for the OH-2 position with OH-2 ≫ OH-3 > OH-6, irrespective of the modification reaction.[3,19] In contrast, it has been shown that the distribution of cationic groups at the polymer level is influenced by different modification reactions. Wilke and Mischnick[3] reported that modification of starch under homogeneous conditions resulted in a more random distribution of cationic groups along the polymer chains than in starch cationized in granular suspension. Furthermore, it has been suggested that dry-cationized starch is mainly substituted at the granule surface, whereas starch modified in suspension has a more uniform distribution of cationic groups through the entire granule.[7,14,20]

In this work, the substituent distribution in cationic potato amylopectin starch (amylose-deficient), modified under three different conditions, was investigated and compared in order to study possible correlations between the modification reaction and the substitution pattern in the final product. The strategy was to hydrolyze the cationic starch samples successively with several enzymes having different selectivities. Subsequent characterization of the products released from each hydrolysis step gave information on the location of substituents in the amylopectin molecule. Furthermore, the use of MS techniques, such as electrospray ionization (ESI)-MS and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS for identification of substituted hydrolysis products was studied.

EXPERIMENTAL SECTION

Substrates. Potato amylopectin starch (PAP) and cationized PAP modified in granular suspension (CPAPg), in solution (CPAPs), and in dry state (CPAPd) were gifts from Lyckeby Stärkelsen (Kristianstad, Sweden). CPAPg was prepared by mixing native PAP in water and Na_2SO_4 (5% w/w). The modification reagent, 2,3-epoxypropyltrimethylammonium chloride, was added and the reaction allowed to proceed under alkaline conditions for 24 h. The reaction mixture was neutralized before washing and drying the products. CPAPs was prepared by gelatinizing native PAP in water at 95 °C before addition of the modification reagent and treatment as above. The product was precipitated in ethanol and finally washed. Preparation of CPAPd was carried out by spraying the modification reagent onto "dry" (as is, 12−20% water content) native PAP and an alkaline activator under vigorous mixing. The reaction proceeded for 72 h at 40 °C, followed by neutralization of the product. The degree of substitution (DS) was calculated from the nitrogen content according to the Kjeldahl method.[21] The water used in all experiments was purified in a Milli-Q system, Millipore (Bedford, MA).

Enzymes. α-Amylase (EC 3.2.1.1) from *Aspergillus oryzae* (Catalog No. E-ANAAM), *β*-amylase (EC 3.2.1.2) from barley (Catalog No. E-BARBP), amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger* (Catalog No. E-AMGPU), and pullulanase (EC 3.2.1.41) from *Klebsiella pneumoniae* (Catalog No. E-PULKP) were obtained from Megazyme International (Bray, County Wicklow, Ireland).

Enzymic Hydrolysis. Native and cationic starch samples were prepared for enzymic hydrolysis by dissolving unmodified PAP (10 mg) or CPAP samples (20 mg) in water (4 mL) at 100 °C for 30 min. Citrate buffer (0.4 M) was used to adjust the pH of the starch solutions to the optimal value (optimal pH varies depending on the enzyme used). After complete incubation, the solutions were boiled for 10 min in order to stop the enzyme reaction.

Pullulanase Hydrolysis. Debranching of native and cationic starch samples was achieved by adjusting the pH of the PAP and CPAP solutions to 5.0 and then incubating with pullulanase (20 U) at 40 °C for 48 h. Before freeze-drying the starch solutions they were ultrafiltrated in Vivaspin Concentrators (Lot No. 99vs0239, Vivascience, Binbrook Hill, Binbrook Lincoln, U.K.) having a membrane with a molecular weight cutoff (MWCO) of 25 000, for removal of the enzymes. Finally, the debranching products obtained were analyzed by ESI-MS and MALDI-MS.

***β*-Amylase Hydrolysis.** Dissolved PAP and CPAP samples were hydrolyzed by *β*-amylase by adjusting the pH of the starch solutions to 6.0 before incubation with *β*-amylase (10 U) at 60 °C for 48 h. The enzyme reaction was stopped, and the amount of maltose released from enzymic hydrolysis was determined with the conventional copper sulfate method.[22,23] The *β*-limit value was calculated as the ratio of maltose liberated from *β*-amylase hydrolysis and the total content of maltose in the starch before hydrolysis.

Amyloglucosidase Hydrolysis. Dissolved PAP and CPAP samples were hydrolyzed by amyloglucosidase by adjusting the pH of the starch solutions to 4.0 and then incubating with amyloglucosidase (30 U) at 40 °C for 48 h. After deactivation of the enzyme, the amount of glucose liberated from the hydrolysis was determined by an enzymic/UV glucose test kit from Megazyme (Catalog No. R-GLC4).

Successive α-Amylase and Amyloglucosidase Hydrolysis. PAP and CPAP starch solutions were given a pH of 5.0 before incubating with α-amylase (30 U) at 40 °C for 24 h. Subsequently, the pH was adjusted to 4.0, amyloglucosidase (30 U) was added, and the starch solutions were incubated for another 24 h. After enzyme deactivation, the amount of glucose liberated from the hydrolysis was determined as described above. In addition, the hydrolysates were investigated by MALDI-MS and ESI-MS. The starch solutions were ultrafiltrated in Vivaspin Concentrators

(15) Solarek, D. B. In *Modified Starches: Properties and Uses*; Wurtzburg, O. B., Ed.; CRC Press: Boca Raton, FL, 1986; pp 113−129.
(16) Kweon, M. R.; Bhirud, P. R.; Sosulski, F. W. *Starch/Stärke* **1996**, *48*, 214−220.
(17) Hellwig, G.; Bischoff, D.; Rubo, A. *Starch/Stärke* **1992**, *44*, 69−74.
(18) Khalil, M. I.; Farag, S. *Starch/Stärke* **1998**, *50*, 267−271.
(19) Wilke, O.; Mischnick, P. *Carbohydr. Res.* **1995**, *275*, 309−318.
(20) Vihervaara, T.; Bruun, H. H.; Backman, R.; Paakkanen, M. *Starch/Stärke* **1990**, *42*, 64−68.
(21) Bradstreet, R. B. *The Kjeldahl Method for Organic Nitrogen*; Academic Press: New York, 1965.
(22) Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19−23.
(23) Nelson, N. *J. Biol. Chem.* **1944**, *153*, 375−380.

Table 1. Various Characteristic Parameters of Native (PAP) and Cationized (CPAP) Potato Amylopectin Starch Obtained from Enzymic Hydrolysis[a]

parameter	PAP	CPAPd	CPAPg	CPAPs
degree of substitution		0.11	0.10	0.14
β-limit value (%)	55.0 (0.2)	16.0 (0.69)	12.8 (0.06)	11.2 (0.07)
glucose liberated (%)[b]				
AMG hydrolysis	96.9 (0.9)	25.1 (0.6)	19.6 (0.2)	16.9 (0.1)
α-amylase + AMG hydrolysis	99.2 (1.2)	53.3 (1.1)	48.2 (0.6)	35.8 (0.7)
pullulanase + α-amylase + AMG hydrolysis	99.7 (0.8)	56.0 (0.1)	51.4 (0.8)	42.2 (1.7)

[a] Mean values ($n = 3$); standard deviations are given within parentheses. [b] The relative amount of glucose liberated is referred to unsubstituted anhydroglucose units.

(Vivascience, MWCO of 25 000) for removal of the enzymes and freeze-dried before the MS analysis.

Successive α-Amylase, Amyloglucosidase, and Pullulanase Hydrolysis. PAP and CPAP starch solutions were adjusted to a pH of 5.0 and then incubated with α-amylase (30 U) at 40 °C for 24 h. The pH was adjusted to 4.0 and amyloglucosidase (30 U) was added, after which the incubation proceeded for another 24 h. Subsequently, the pH was readjusted to 5.0, pullulanase (20 U) was added, and the solutions were further incubated for 24 h. Finally, the enzyme reaction was stopped and the amount of glucose liberated from the hydrolysis was determined as described above.

Electrospray Mass Spectrometry. Mass spectrometry analysis of enzymic hydrolysates of PAP and CPAP starch samples was performed on an Esquire-LC mass spectrometer from Bruker Daltonik GmbH (Bremen, Germany), equipped with an ESI interface and a quadrupole ion trap mass analyzer. The mass spectrometer was typically 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 nebulizer gas at a pressure of 30 psi 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 Inc. (Haverhill, MA). The spectra were acquired in the positive ion mode. Full-scan spectra were acquired in the mass-to-charge ratio (m/z) range of 50−2200, whereas weak signal species were detected using a scan window of m/z 100. Before injection into the ESI interface, the freeze-dried PAP and CPAP hydrolysates were dissolved in water/methanol (50% v/v) to a concentration of 5 mg/mL. The samples were continuously introduced into the interface by a syringe pump (Cole Parmer 74900 series, Cole-Parmer Instrument Co., Vernon Hills, IL) equipped with a 250-μL syringe at a flow rate of 1.5 μL/min.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. MALDI-TOF-MS experiments were performed on a PerSeptive Voyager-DE STR (Applied Biosystems, Framingham, MA) equipped with a N_2 laser, time-lag focusing, reflector, and a tandem coupled microchannel plate detector in reflector mode. All mass spectra were acquired in reflector mode. The accelerating voltage was 20 kV, and the reflector voltage was 13% higher. The laser intensity was held slightly above threshold, and the lag time was 150 ns plus instrument offset. To prevent saturation of the detector, the mass gate was held at m/z 300. The mass spectra were accumulated from 100 laser shots and acquired in positive ion mode.

2,5-Dihydroxybenzoic acid was used for the analysis of CPAP. The matrix was dissolved (10 g/L) in H_2O and the analyte (1 g/L) in H_2O. The matrix and analyte solutions were then mixed 4:1, and 1 μL of the mixture was applied on a MALDI sample plate and allowed to dry at ambient atmosphere.

RESULTS AND DISCUSSION

The location of quaternary ammonium groups in the three cationic starch samples, modified in granular slurry (CPAPg), dry state (CPAPd), and solution (CPAPs), were investigated by means of single or consecutive enzymic hydrolysis with four different starch-hydrolyzing enzymes: pullulanase, α-amylase, β-amylase, and amyloglucosidase. Each of these enzymes has different selectivities on the glucosidic linkages in native starch that are well-known and well-documented. In order to elucidate the substituent distribution in cationic starch, it was assumed that the enzymic hydrolysis of glucosidic linkages is inhibited by the quaternary ammonium groups. Consequently, the product spectrum obtained from enzymic hydrolysis of modified starch differs from that of native, unmodified starch. Furthermore, as the enzymes used have different hydrolytic actions on starch, each of these enzymes gives specific information on the distribution of cationic groups in the amylopectin molecule.

***β*-Amylase Hydrolysis.** β-Amylase is an exoenzyme that starts acting from the nonreducing end. It catalyses the hydrolysis of every second α-(1 → 4) D-glucosidic linkage until it reaches a branching point, where it stops. This action results in production of β-maltose and a remaining β-limit dextrin.[24] As β-amylase acts upon the exterior chains, that is, the chains located outside the branching points, native PAP and CPAP samples were hydrolyzed with β-amylase in order to get information on the distribution of cationic groups in the outer regions of the amylopectin molecule and in the nonreducing ends of the chains.

The β-limit values obtained are presented in Table 1, where it can be seen that native PAP was hydrolyzed to 55% by β-amylase, which is in accordance with previously reported results.[25,26] However, the CPAP samples were hydrolyzed to a lesser extent, due to the cationic groups located on the exterior chains that hinder the enzyme action. The CPAPd showed the highest β-limit value (16.0%) and the CPAPs the lowest (11.2%). As β-amylase

(24) Guilbot, A.; Mercier, C. In *The Polysaccharides*; Aspinall, G., Ed.; Academic Press: New York, 1985; Vol. 3, pp 209−273.
(25) Praznik, W.; Rammesmayer, G.; Spies, T.; Huber, A. *Carbohydr. Res.* **1992**, *227*, 171−182.
(26) Nilsson, G.; Richardson, S.; Huber, A.; Torto, N.; Laurell, T.; Gorton, L. *Carbohydr. Polym.* **2001**, *46*, 59−68.

obviously hydrolyses the former sample somewhat more than the latter, this result implies that the cationic groups are located slightly closer to the branching points in CPAPd, whereas in CPAPs, the substituents are to be found further out on the exterior chains. This could indicate that the reactivity of the regions in the vicinity of the branching points is higher in CPAPd than in CPAPs, which can be explained by the modification conditions. The CPAPd sample has been modified in a dry state, where the semicrystalline structure of amylopectin, with alternating crystalline and amorphous zones, remains intact, whereas the CPAPs sample was dissolved and the crystallinity destroyed before adding the modification reagents. Thus, the CPAPs sample exhibits a more uniform reactivity over the entire amylopectin molecule than does the CPAPd sample, which may have a slightly higher reactivity in the amorphous regions around the branching points. The results obtained here are in accordance with previous studies on other starch derivatives,[4,10,27] which showed that substitution of starch takes place preferentially in the amorphous regions. There was no significant difference between the β-limit values of CPAPs and CPAPg, thus suggesting that the substitution of the exterior chains in these samples was similar.

Amyloglucosidase Hydrolysis. Amyloglucosidase is an exoenzyme that starts from the nonreducing ends and hydrolyses every successive α-(1 → 4)- and α-(1→ 6) D-glucosidic linkage in starch, with the release of glucose.[24]

Amyloglucosidase hydrolysis of the CPAP samples, followed by determination of the amount of glucose released, yielded additional information on the substitution around the branching points. If all exterior chains carry at least one cationic group, the degree of hydrolysis of CPAP by β-amylase or amyloglucosidase would be the same (assuming that the cationic groups hinder both enzymes to the same extent). According to the results obtained (Table 1), amyloglucosidase hydrolyzed the cationic starch samples to a significantly higher extent than did β-amylase. This large difference in degree of hydrolysis suggests that there must exist exterior chains, including the branching points, which are unsubstituted. (The CPAPd sample showed the highest difference in degree of hydrolysis between β-amylase and amyloglucosidase hydrolysis, thus indicating that this sample contains more unsubstituted chains with unsubstituted branching points than CPAPg and CPAPs.) This finding is somewhat contradictory, as dry cationization is expected to result in a slight concentration of cationic groups around the branching points due to a higher reactivity in the amorphous areas than in the crystalline regions. The result obtained here indicates the opposite, that more branching points are unsubstituted in CPAPd compared with CPAPs and CPAPg. Manelius et al. have suggested that dry-cationized potato starch is preferentially substituted at the granular surface, whereas the interior parts of the granules remain virtually unsubstituted.[7] A higher local concentration of cationic components in dry-cationized starch implies that more material is accessible for enzymic attack. Thus, one could speculate that CPAPd is degraded to a higher extent by amyloglucosidase than is CPAPs and CPAPg not because of more unsubstituted branching points, but because of higher concentration of unsubstituted material in certain regions in the granule.

Successive α-Amylase and Amyloglucosidase Hydrolysis. An interesting parameter when the substituent distribution is studied is the heterogeneity/homogeneity of substitution, that is, if the cationic groups are, for example, randomly or blockwise distributed. This can be investigated by exhaustive enzymic hydrolysis of modified starch, followed by determination of the amount of glucose liberated. The more glucose released from a modified starch sample with a given DS, the more heterogeneous is the distribution of substituent groups, as the average length of sequences of adjacent unsubstituted glucose units, where the enzymes can get access to the glucosidic linkages, is longer when the substituents are more clustered. This statement is based on the assumption that only glucosidic linkages between two unsubstituted glucose units are susceptible to enzymic attack.

To achieve as complete hydrolysis as possible, the CPAP samples were subjected to successive α-amylase and amyloglucosidase hydrolysis. α-Amylase is an endoenzyme that randomly catalyses the hydrolysis of α-(1 → 4) D-glucosidic linkages inside the starch chain and thereby liberating new nonreducing ends, which facilitates the subsequent amyloglucosidase attack.[24]

As expected, native PAP was completely hydrolyzed to glucose by the successive action of α-amylase and amyloglucosidase (Table 1). In the hydrolysis of the CPAP samples, the ability of the enzymes to cleave the glucosidic linkages was reduced due to the cationic groups. This hindrance was most pronounced in CPAPs and least in CPAPd (Table 1), indicating that the former sample had the most homogeneous distribution. The more heterogeneous distribution of cationic groups in CPAPd could be explained by the remaining crystalline and amorphous structures in the amylopectin molecule, where the amorphous regions exhibit higher reactivity, thus resulting in a more heterogeneous distribution of cationic groups than in CPAPs (without crystallinity). This result is in agreement with the results obtained from the β-amylase hydrolysis, which indicated that the substitution in CPAPd occurs slightly closer to the amorphous areas around the branching points than does the substitution in CPAPs. Further, the result supports the theory discussed in the section above, where it is suggested that starch cationized in solution leads to a roughly uniform distribution of substituents in the entire granule, whereas dry-cationized starch is preferably substituted in the outer parts of the granule.[7,14,20] CPAPg appeared to have a distribution of cationic groups with a heterogeneity between the CPAPs and CPAPd samples. CPAPg is produced in a modification reaction that takes place in a slurry of granules, where most of the granules are supposed to be intact. However, a minor part of the granules is thought to be disrupted, resulting in a slight decrease of the crystallinity. Consequently, in comparison with CPAPd with intact crystalline areas, the reactivity is slightly more uniform over the entire CPAPg sample, thus ending up in a more homogeneous substituent distribution. On the other hand, CPAPs, with destroyed crystallinity, exhibits a more uniform reactivity than does CPAPg, which explains the suggestion of a more homogeneous distribution of cationic groups in the former sample.

Successive α-Amylase, Amyloglucosidase, and Pullulanase Hydrolysis. For further investigation of the substitution around the branching points, the CPAP samples were successively hydrolyzed by α-amylase, amyloglucosidase, and finally pullulanase, a debranching enzyme that catalyzes the hydrolysis of α-(1

(27) Zhu, Q.; Sjöholm, R.; Nurmi, K.; Bertoft, E. *Carbohydr. Res.* **1998**, *309*, 213-218.

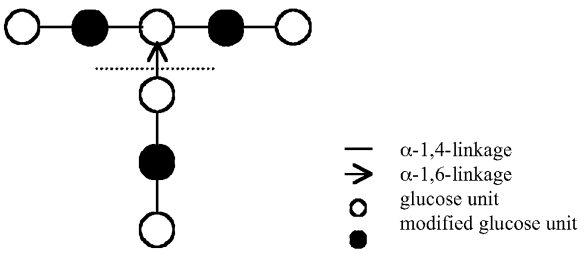

Figure 1. Cationized oligosaccharide that is a possible substrate for pullulanase hydrolysis but not for amyloglucosidase.

→ 6) D-glucosidic linkages in amylopectin.[24] The results in Table 1 show that the addition of pullulanase to an α-amylase and amyloglucosidase hydrolysate leads to further degradation, although slight, of the oligomeric products obtained from the α-amylase and amyloglucosidase hydrolysis, as a higher amount of glucose is measured after the final pullulanase hydrolysis step. Obviously, there exist α-(1 → 6) D-glucosidic linkages only accessible for pullulanase but not amyloglucosidase hydrolysis. These α-(1 → 6) linkages should be located between two unsubstituted glucose units but otherwise surrounded by substituted glucose units (assuming that both enzymes cleave linkages only next to unsubstituted glucose units). The structure of such a substrate is suggested in Figure 1.

The highest relative difference in amount of glucose liberated before and after pullulanase hydrolysis was found in CPAPs, which indicates that CPAPs has the highest number of unsubstituted branching points and therefore the lowest degree of substitution around the (1 → 6) linkages. This agrees with the discussion that CPAPs, with destroyed crystallinity and therefore a more homogeneous reactivity over the entire molecule, is less substituted in the vicinity to the branching points in comparison with CPAPd, which has a remaining semicrystalline structure and therefore also a higher reactivity in the branching point surroundings. The considerably lower relative increase of released glucose after pullulanase hydrolysis in the CPAPd and CPAPg samples (see Table 1) also points toward a higher concentration of cationic groups at the (1 → 6) linkages in these samples than in CPAPs.

Mass Spectrometry Analysis. In this work, ESI-MS and MALDI-MS were used primarily for analysis of substituted oligosaccharides liberated from enzymic degradation of cationic starch. Correct identification of the oligosaccharides formed upon enzymic degradation of modified starch is crucial in order to characterize the fine structure of the corresponding, intact polysaccharide. Thus, development of techniques that deal with this issue is of vital importance. At present, HPAEC with pulsed amperometric detection is probably the most widely used technique for characterization of oligosaccharides.[28,29] However, the elution order of substituted oligomers is rather unpredictable and commercial standards usually unavailable, making identification of substituted oligosaccharides difficult. As substituted saccharides differ from their unsubstituted counterparts by their mass rather than configuration, MS should be an appropriate choice of method. In a few previous investigations of the substitution pattern in starch and cellulose derivatives or related compounds, MALDI-MS has been employed for identification of enzymic hydrolysis products.[8,30−33] However, none of these studies deal with enzymic hydrolysis of cationic starch. Moreover, no such studies where ESI-MS is employed for investigation of the substituent distribution have been reported up to date to the authors' knowledge, except a recently published work on hydroxypropylated starch carried out on the authors' laboratory.[9]

Electrospray Ionization Mass Spectrometry. The hydrolysates from successive α-amylase and amyloglucosidase hydrolysis of CPAP samples were investigated by ESI-MS with the aim to identify both unsubstituted and substituted products released. As the substituted products were already present as cations (due to the permanently bound quaternary ammonium groups), the problem concerning low ionization efficiency discussed above was circumvented. Unsubstituted products turned out to be analyzed most effectively as sodium adducts, although the signal strength was considerably lower than that of the substituted analytes. Figures 2−4 present the mass spectra from direct injection of the α-amylase and amyloglucosidase hydrolysates of CPAPg, CPAPs, and CPAPd samples. The primary features of these spectra are the strong signals at m/z 620 and 782, which correspond to monosubstituted maltotriose and maltotetraose, respectively. Other distinct signals in the spectra are m/z 458, 530, and 611, which demonstrate the presence of monosubstituted maltose, disubstituted maltopentaose, and disubstituted maltohexaose, respectively, in the hydrolysates. All three spectra are similar to each other; the only obvious difference is that monosubstituted maltotetraose (m/z 782) is the product with the highest relative intensity in the CPAPg hydrolysate, whereas in the CPAPs and CPAPd hydrolysates monosubstituted maltotriose (m/z 620) exhibits the highest relative intensity. This result indicates that monosubstituted maltotetraose is the most abundant substituted oligomer released from α-amylase and amyloglucosidase hydrolysis of CPAPg, whereas monosubstituted maltotriose is the most abundant hydrolysis product of the CPAPs and CPAPg samples. However, one should be extremely cautious when interpreting the relative intensities of the MS signals to directly extract quantitative data without a correct calibration of the instrument with internal standards. As there are no proper standards of these cationic oligomers available, no such calibration can be performed. The hydrolysis products that could be identified from the signals visible in the mass spectra depicted in Figures 2−4 are probably the most abundant components of the enzymic hydrolysates. However, there could be additional hydrolysis products present in the hydrolysates that are not visible in the spectra due to low signal intensities. Therefore, individual compounds were searched for utilizing a narrow scan. This procedure revealed an additional number of hydrolysis products that are presented in Table 2. As seen in the table, the only unsubstituted product obtained is glucose (detected as monosodium adduct, $[M + Na]^+$, at m/z

(28) Lee, Y. C. *J. Chromatogr., A* **1996**, *720*, 137−149.
(29) Cataldi, T. R. I.; Campa, C.; De Benedetto, G. E. *Fresenius J. Anal. Chem.* **2000**, *368*, 739−758.
(30) Mischnick, P. Analytische Charakterisierung von Polysacchariden und Polysaccharidderivaten mittels chemischer, enzymatischer und massenspektrometrischer Methoden. Habilitationsschrift, Universität Hamburg, Germany, 1995.
(31) Daas, P.; Arisz, P.; Schols, H.; De Ruiter, G.; Voragen, A. *Anal. Biochem.* **1998**, *257*, 195−202.
(32) Körner, R.; Limberg, G.; Mikkelsen, J.; Roepstorff, P. *J. Mass Spectrom.* **1998**, *33*, 836−842.
(33) Limberg, G.; Körner, R.; Buchholt, H.; Christensen, T.; Roepstorff, P.; Mikkelsen, J. *Carbohydr. Res.* **2000**, *327*, 293−307.

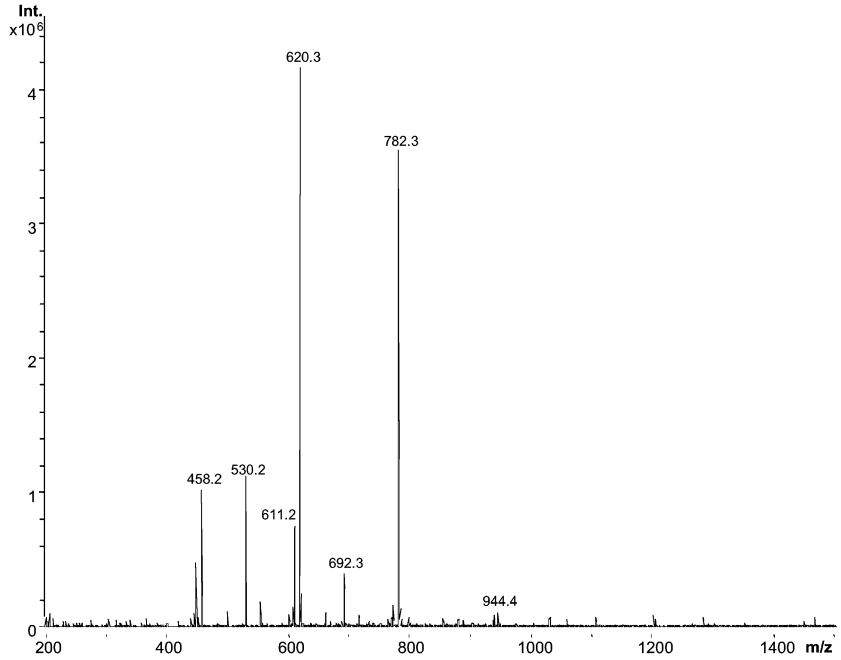

Figure 2. Mass spectrum from the ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of CPAPs.

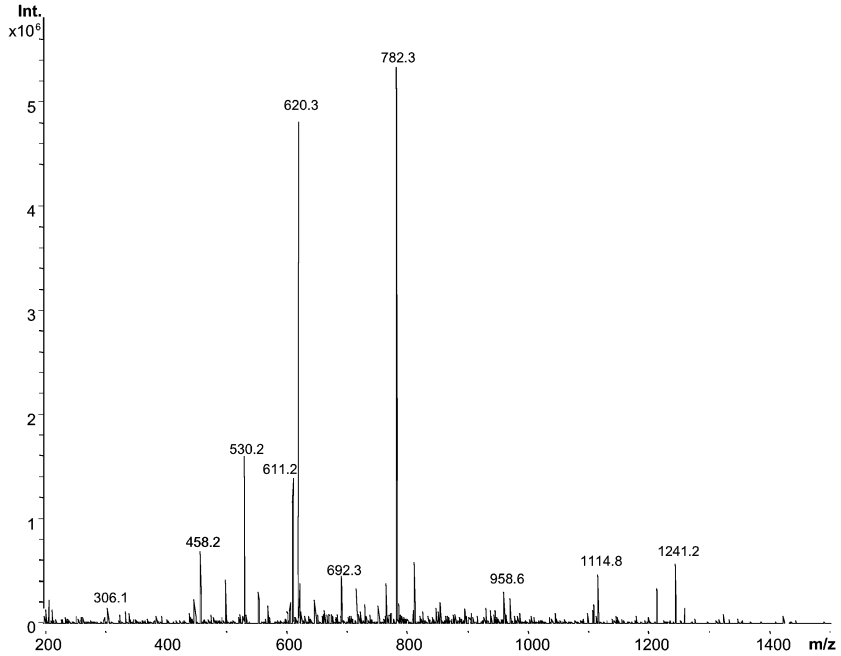

Figure 3. Mass spectrum from the ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of CPAPg.

203). This result is in full agreement with the theoretical enzyme action; unsubstituted oligomers produced, both linear and branched, should be further—and finally—degraded to glucose by the concurrent α-amylase and amyloglucosidase action or the amyloglucosidase action alone. The fact that glucose has a relative ion intensity too low to be seen in the spectra in Figures 2−4, although this is by the far most common hydrolysis product released from the enzymic hydrolysis (35−53% of the CPAP samples are degraded to glucose, see Table 1), demonstrates the large difference in ionization efficiency between sodium adducts of neutral sugars and cationized oligosaccharides with a permanent positive charge. Furthermore, it is a striking example of the above-mentioned statement that the relative ion intensities may be very misleading in quantifications.

The substituted hydrolysis products observed in the mass spectra had a degree of polymerization (DP) from 2 up to 11 (DP 2−DP 11) and were mainly monosubstituted, that is, modified with one quaternary ammonium group, although di-, tri-, and tetrasubstituted oligomers were found as well. As a complementary source of information, ESI tandem mass spectrometry (ESI-MS/MS) was employed for further verification of the identity of each oligomeric product characterized. The MS/MS spectrum of monosubstituted maltotriose (m/z 620) presented in Figure 5 demonstrates the characteristic mass losses of 60 ($C_2H_4O_2$) and 120 Da ($C_4H_8O_4$), which corresponds to cross-ring cleavages of the oligosaccharide.[34,35] The presence of the −60-Da product ion in combination with the absence of a −90-Da product ion is indicative of (1 → 4) glucosidic linkages.[35] The fragment ions at m/z 440 (loss of 180)

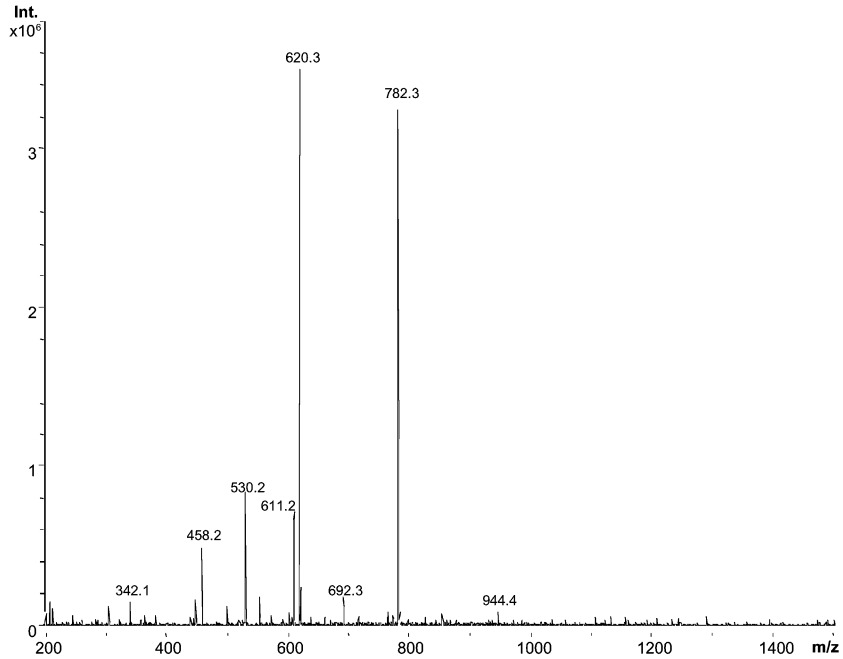

Figure 4. Mass spectrum from the ESI-MS analysis of α-amylase and amyloglucosidase hydrolysate of CPAPd.

Table 2. Products Obtained from Successive α-Amylase and Amyloglucosidase Hydrolysis of CPAP Samples Detected by ESI-MS

product[a]	*m*/*z*	CPAPd	CPAPg	CPAPs
*n*1	203	X	X	X
*n*2 + *r*1	458	X	X	X
*n*3 + *r*1	620	X	X	X
*n*3 + *r*2	368	X		X
*n*4 + *r*1	782	X	X	X
*n*4 + *r*2	449	X	X	X
*n*5 + *r*1	944	X	X	X
*n*5 + *r*2	530	X	X	X
*n*5 + *r*3	392	X	X	X
*n*6 + *r*1	1106	X	X	X
*n*6 + *r*2	611	X	X	X
*n*6 + *r*3	446	X	X	X
*n*7 + *r*2	692	X	X	X
*n*7 + *r*3	500	X	X	X
*n*8 + *r*2	773	X		
*n*8 + *r*3	554	X	X	X
*n*8 + *r*4	444.5			X
*n*9 + *r*3	608	X	X	
*n*9 + *r*4	485	X	X	
*n*10 + *r*3	662	X	X	X
*n*10 + *r*4	525.5	X	X	
*n*11 + *r*4	566	X	X	

[a] *n*, number of glucose units; *r*, number of substituents.

and 278 (loss of 2 × 180) originate from cleavage on one side of the glucosidic bond, whereas the weak signals at *m*/*z* 458 (loss of 162) and 296 (loss of 2 × 162) are derived from cleavage on the other side of the bond.

Considering the distribution of cationic groups according to the results obtained from ESI-MS analysis, it was suggested that the substituents were randomly distributed in the amylopectin molecule as only low-substituted oligomers were observed. No longer, highly substituted products indicating a more heterogeneous distribution could be found. The hydrolysis product of highest DS detected was very small amounts of trisubstituted maltopentaose, which is hardly evidence of heterogeneity in the distribution. Furthermore, in this particular case, it is highly unlikely to get highly substituted oligomers even for a heterogeneous distribution, as the samples have such low DS (0.11−0.14). There were no pronounced differences in the product spectrum between the three CPAP samples modified under different conditions, although the occurrence of longer oligomers (DP 9−DP 11) was less frequent in the spectrum of the CPAPs hydrolysate (see Table 2). A detailed study of the substituted products obtained (see Table 2) revealed that both enzymes preferentially hydrolyze linkages adjacent to unsubstituted glucose units, as no substituted monomers were detected. In general, starch-hydrolyzing enzymes exhibit strong preference for glucosidic linkages between unsubstituted residues, and the ability to attack linkages next to substituted glucose units depends on parameters such as size, polarity, and position of the substituent in the AGU. In addition, the selectivity of the enzyme and the rate of hydrolysis are influenced by the number of subsites and the shape of the active site, which vary depending on the enzyme origin/source.[5,12,36] However, the peak observed at *m*/*z* 458 corresponding to maltose substituted with one quaternary ammonium group suggests that one or both of the enzymes do have the capacity to cleave glucosidic linkages next to substituted glucose residues, although this capacity seems to be significantly reduced. An alternative explanation is that the monosubstituted maltose originates from the reducing ends and the quaternary ammonium group is bound to the reducing glucose residue; in this case, it could imply that no linkages next to cationized AGUs have been hydrolyzed. The presence of monosubstituted DP 4−6

(34) Garozzo, D.; Giuffrida, M.; Impallomeni, G.; Ballistreri, A.; Montaudo, G. *Anal. Chem.* **1990**, *62*, 279−286.
(35) Asam, M. R.; Glish, G. L. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 987−995.
(36) Roesser, D. S.; McCarthy, S. P.; Gross, R. A.; Kaplan, D. L. *Macromolecules* **1996**, *29*, 1−9.

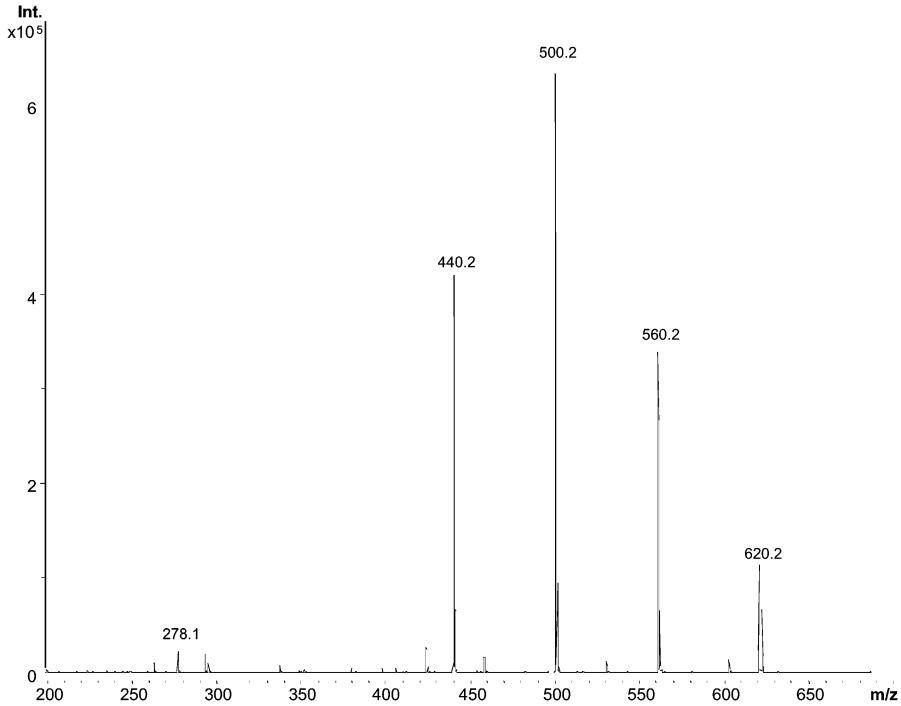

Figure 5. MS/MS spectrum of monosubstituted maltotriose (*m*/*z* 620), demonstrating the characteristic mass losses.

and disubstituted DP 5−8 oligomers suggests that the hydrolytic action of the enzymes is reduced even when there are two or more adjoining unsubstituted glucose residues between a glucosidic bond. This finding could be explained by the fact that the quaternary ammonium group is a very bulky substituent, hence providing a large steric hindrance to enzyme attack.

ESI-MS and MS^n are useful techniques for analyzing enzymic hydrolysates. ESI is a soft ionization technique that allows ionization of sugar compounds without any fragmentation, unless you choose a controlled fragmentation by MS^n analysis. The purity requirements are lowered, as the possibility to scan a very narrow *m*/*z* range increases the sensitivity markedly. Additionally, the cationic oligomers studied in this work were found to be very appropriate for ESI-MS analysis due to the permanently bound positive charge, giving ions of high abundance. The main drawback of the technique is the difficulty in acquiring correct quantitative data, due to the lack of proper standards.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. The MALDI mass spectra of substituted products liberated from successive α-amylase and amyloglucosidase hydrolysis of the CPAP samples are shown in Figure 6d−f. The hydrolysis products were detected in their native state, that is, as cations, due to the permanent positive charge on the quaternary ammonium group. No ions corresponding to sodium or potassium adducts of the oligomeric products were found in the spectra. According to the results obtained from the MALDI-MS analysis, the enzymic hydrolysates of the CPAP samples contained mono-, di-, and trisubstituted products of DP 2−DP 10 (see Table 3), whereas no unmodified oligosaccharides were detected. The absence of unmodified products is most likely due to the fact that unmodified oligomers released are immediately further hydrolyzed to glucose by the enzymes, although one has to consider the risk that the ionization efficiency of neutral saccharides is too low to yield detectable amounts of ions. Moreover, ions with *m*/*z* below 400 were omitted in the acquisitions as the very intense matrix signal in this region reduces the efficiency of the detector, thus influencing the signal intensity of the higher *m*/*z* portion negatively.When studying the substituted products in the spectra, it was found that the desorption/ionization process caused an elimination of trimethylamine from one of the *O*-(2-hydroxy-3-methylammonium)propyl groups in disubstituted oligomers and from two of the *O*-(2-hydroxy-3-methylammonium)-propyl groups in trisubstituted oligomers (see Figure 7). Therefore, all disubstituted products were detected as singly charged oligomers with one *O*-(2-hydroxy-3-methylammonium)propyl residue and one residual *O*-(2-oxo)propyl group, which is equivalent to a mass increment of 56 on the *m*/*z* value of the corresponding monosubstituted oligomer. Similarly, trisubstituted products were detected as singly charged oligomers with one *O*-(2-hydroxy-3-methylammonium)propyl residue and two residual *O*-(2-oxo)-propyl groups. This is in accordance with an earlier study on cationic amylose, where it was shown that oligomers with multiple cationic substituents lost trimethylamine residues until only one cationic group was left on the molecule.[30] In Table 3 it can be seen that mono-, di-, and trisubstituted products were detected in the α-amylase and amyloglucosidase hydrolysates of the CPAP samples. Although fewer trisubstituted and no tetrasubstituted oligomers were present in the MALDI mass spectra, the results are similar to those obtained by the ESI-MS analysis and no additional information on the heterogeneity of the substituent distribution could be obtained.

In Figure 6a−c, the MALDI mass spectra from analysis of pullulanase digests of the CPAP samples are shown. The major unit chain distribution of the debranched CPAP samples seen in the spectra is derived from monosubstituted chains of DP 6 up to approximately DP 23. There is also a less significant distribution corresponding to disubstituted chains of DP 7−DP 23, detected as singly charged oligomers with one intact *O*-(2-hydroxy-3-

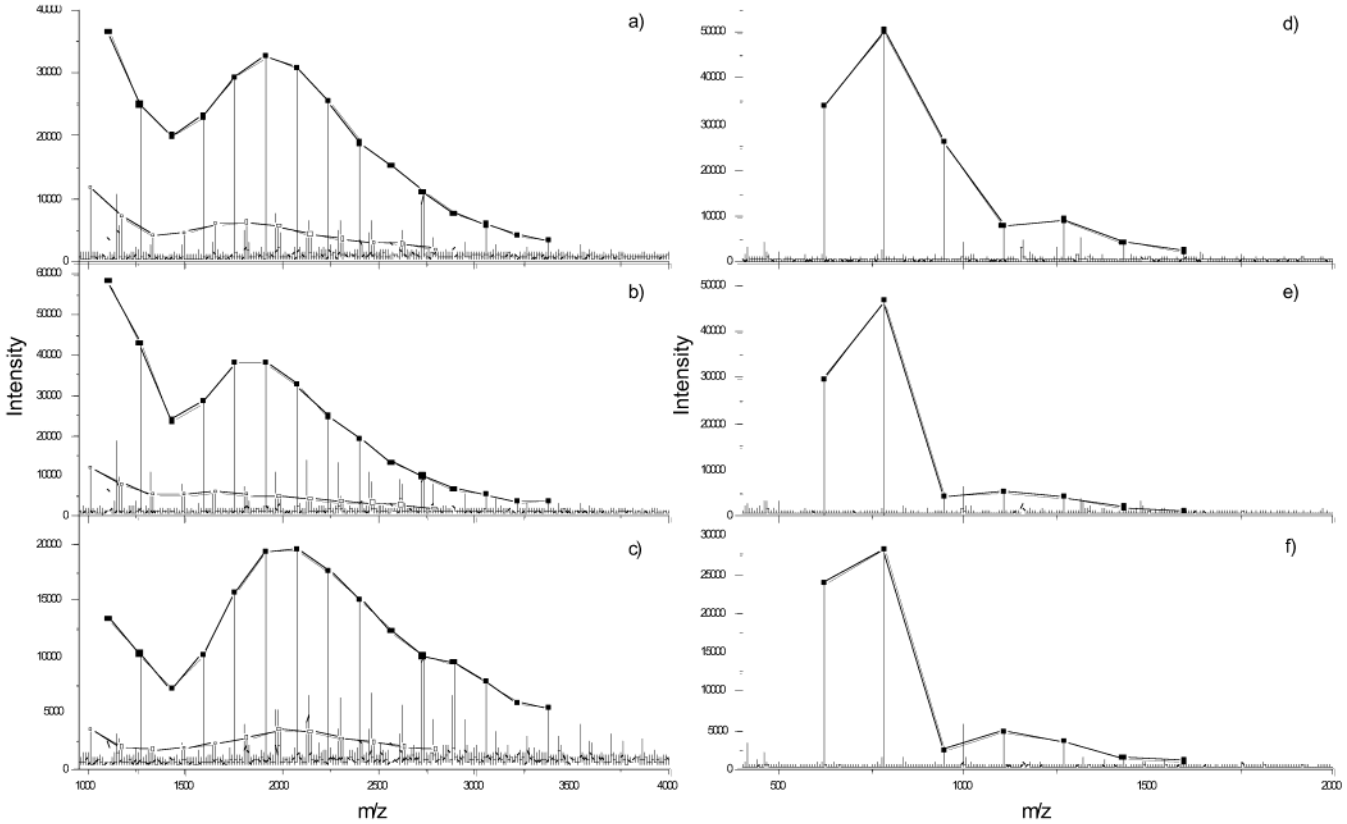

Figure 6. Figure 6. Reflector mode MALDI-TOF mass spectra of pullulanase hydrolysates of (a) CPAPg, (b) CPAPs, and (c) CPAPd and α-amylase and amyloglucosidase hydrolysates of (d) CPAPg, (e) CPAPs, and (f) CPAPd. The lines connected with filled boxes indicate molecules containing one modification. In spectra a−c, oligosaccharides with DP = 6−20 containing one modification are indicated, and in spectra d−f, the corresponding range is DP = 3−9. The lines connected with empty boxes in spectra a−c indicate the unmodified oligosaccharides with DP = 6−17. Not all detected DPs are indicated in the mass spectra.

Table 3. Products Obtained from Successive α-Amylase and Amyloglucosidase Hydrolysis of CPAP Samples Detected by MALDI-MS

product[a]	*m*/*z*	CPAPd	CPAPg	CPAPs
*n*2 + *r*1	458	X	X	X
*n*3 + *r*1	620	X	X	X
*n*4 + *r*1	782	X	X	X
*n*4 + *r*2	838	X	X	X
*n*5 + *r*1	944	X	X	X
*n*5 + *r*2	1000	X	X	X
*n*6 + *r*1	1106	X	X	X
*n*6 + *r*2	1162	X	X	X
*n*7 + *r*1	1268	X	X	X
*n*7 + *r*2	1324	X	X	
*n*7 + *r*3	1380	X	X	X
*n*8 + *r*1	1430	X	X	X
*n*8 + *r*2	1486	X	X	X
*n*8 + *r*3	1542	X		X
*n*9 + *r*1	1592	X	X	
*n*9 + *r*2	1648	X	X	X
*n*9 + *r*3	1704	X		
*n*10 + *r*1	1754	X	X	
*n*10 + *r*2	1810	X		
*n*10 + *r*3	1866	X		

[a] *n*, number of glucose units; *r*, number of substituents.

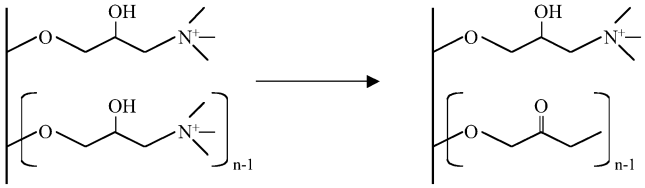

Figure 7. Elimination of trimethylamine from an *O*-(2-hydroxy-3-methylammonium)propyl group.

methylammonium)propyl residue and one residual *O*-(2-oxo)-propyl group. Among the largest products visible in the spectra, it is also possible to detect chains carrying three substituents. In addition, there is a third (minor) chain distribution observed in the spectra, that originates from unmodified debranching products of DP 6−DP 17. These oligomers without any charged, covalently bound substitution groups are present and determined as sodium adducts, $[M + Na]^+$. According to the results obtained from the MALDI-MS analysis, the debranched chains of DP 6−23 are un-, mono-, di-, or trisubstituted. It might at first seem somewhat notable that even the longer chains carry not more than three cationic groups, but considering the DS (0.11−0.14) and the average chain length (25.6 glucose units[4]) of the CPAP samples, this is actually an expected result, at least for a homogeneous distribution of substituents.

The conclusion is that there is no evidence of clustering of cationic groups in the CPAP samples. On the other hand, the presence of unmodified chains indicates that the distribution is not completely statistically random either. No significant difference in the chain length distribution could be found between the CPAP samples modified under different conditions; all three spectra (Figure 6a−c) revealed almost the same debranching products.

It would have been very valuable to have a thorough quantitative analysis of these results. However, accurate quantification requires internal standard compounds, which are not available for cationic oligomers. The signal intensity depends on several factors such as matrix, buffer salts, and heterogeneity of the target.[37,38] Furthermore, differences in chemical properties and molecular weights within the samples give rise to discrimination effects, which complicate the quantitative determination.[39,40] Consequently, neither estimations of the relative amount of unmodified chain compared with modified chains, nor monosubstituted compared with disubstituted chains, nor individual unit chains in the spectra could be made.

The MALDI-MS analysis was informative when the enzymic (α-amylase and amyloglucosidase) action on cationic starch was studied, although one obvious limitation is the reduced capability to detect analyte molecules in the lower mass range. According to the results obtained in the MALDI-MS analysis (see Table 3), the successive hydrolysis of cationic starch by α-amylase and amyloglucosidase produced disubstituted oligomers of DP 4 up to DP 10 and trisubstituted products of DP 7−DP 10. This result suggests that the enzymes have preference for glucosidic linkages between unsubstituted glucose units. However, the presence of monosubstituted products of DP 4−DP 10 indicates that two adjacent unsubstituted glucose units in many cases are not enough for hydrolysis to occur. These findings about the enzyme action confirm the conclusions drawn from the ESI-MS analysis. As it is difficult to detect analytes in the lower mass range, less detailed information on the enzymic hydrolysis could be obtained with MALDI-MS than with ESI-MS. Furthermore, MS/MS experiments were not attempted.

CONCLUSIONS

Successive enzymic hydrolysis of cationic potato amylopectin starch samples, modified under different conditions, followed by analysis of the hydrolysis products obtained, revealed slight differences in the distribution of cationic groups along the polymer chain between the samples. All three samples were found to have a more or less homogeneous distribution, with the sample modified in solution the most, and the sample modified in dry state the least homogeneous. It was demonstrated that dry-cationized amylopectin had a slight tendency for clustering of cationic groups around the branching points compared with amylopectin modified in granular slurry or solution. The higher reactivity in the regions around the branching points is explained by the intact crystalline and amorphous areas in the former sample. The use of several enzymes with different selectivities was found to give different kinds of, and also complementary, information on the substituent distribution.

ESI-MS and MALDI-MS were both found to be very useful techniques for identification of substituted products released from enzymic hydrolysis of cationized amylopectin starch. It was demonstrated that ESI-MS is more efficient for analysis of low molecular mass compounds and therefore more appropriate when the enzyme action on the cationic starch samples was studied. ESI-MS allowed characterization of hydrolysis products with up to four substitution groups, whereas the MALDI-MS detected oligomers substituted with up to three substituents. ESI-MS/MS was successfully employed for detailed structural identification of the substituted oligomers. On the other hand, MALDI-MS was successfully employed for analysis of the debranching products obtained from pullulanase hydrolysis of cationic starch. Unfortunately, no quantitative data could be obtained from either ESI-MS or MALDI-MS due to the lack of reference compounds for the cationic oligomers.

(37) Mock, K. K.; Sutton, C. W.; Cottrell, J. S. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 223−238.
(38) Naven, T. J. P.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1361−1366.
(39) Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 614−619.
(40) Harvey, D. J.; Kuster, B.; Naven, T. J. P. *Glycoconjugate J.* **1998**, *15*, 333−338.

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