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Synthesis and application of epoxy starch derivatives

Annemarie M.L. Huijbrechts [a,b,*,1], Ruud ter Haar [c], Henk A. Schols [c,1], Maurice C.R. Franssen [a], Carmen G. Boeriu [b,d,1], Ernst J.R. Sudhölter [e]

[a] Laboratory of Organic Chemistry, Wageningen University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands
[b] Division of Biobased Products, Agrotechnology and Food Sciences Group, Bornsesteeg 59, 6708 PD Wageningen, The Netherlands
[c] Laboratory of Food Chemistry, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands
[d] University "Aurel Vlaicu", Department of Chemical and Biological Sciences, Arad, Romania
[e] Delft University of Technology, Laboratory of Nano-Organic Chemistry, Department of Chemical Engineering 'DelftChemTech', Julianalaan 136, 2628 BL, Delft, The Netherlands

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ABSTRACT

Epoxy starch derivatives were synthesized by epoxidation of allylated starch. The reaction was performed with low substituted 1-allyloxy-2-hydroxypropyl-waxy maize starch (AHP-WMS; degree of substitution (DS) of 0.23) using hydrogen peroxide and acetonitrile Via a two step spectrophotometric assay, it was determined that epoxy-WMS contained 0.13 ± 0.03 mmol epoxy groups per gram dry allylated starch which corresponds to DS value of 0.025. Enzymatic digestibility, swelling capacity and solubility were significantly reduced after epoxidation. The detailed chemical structure of epoxy-WMS was characterized by enzymatic hydrolysis followed by chromatographic and mass spectrometric techniques. Only a small amount of epoxidized oligomers was found in the enzymatic digested products of epoxy-WMS. Apparently, the epoxidation reaction is highly efficient but subsequent reactions of epoxy groups lead to a considerable amount of cross-links and diol groups. Additionally, epoxy starch derivatives were successfully applied as carrier matrix for immobilization of an enzyme.

1. Introduction

Because of biocompatibility, degradability and low costs, starch and starch derivatives are used as excipients for tabletting and matrices for delivery systems in pharmaceutical industry (Dumoulin, Cartilier, & Mateescu, 1999; Röper, 2002). There is a strong interest in chemically modified starch derivatives possessing functional groups that allow easy binding of ligands to the surface. Amongst these starch products are derivatives possessing epoxy functions (Bai, Li, & Wang, 2006; Lin & Huang, 1992; Maher, 1977; Slaghek, Timmermans, & Gotlieb, 2001). These reactive epoxy groups allow easy binding of ligands such as polyamines, peptides and amino acids. Moreover, epoxy groups can advantageously serve as cross-linker in starch. Hence, these starch derivatives can be used in coatings and stabilizers, but they can also serve as carriers for delivery systems and controlled drugs release.

Incorporation of epoxy groups into carbohydrates can be performed directly (Burton & Harding, 1997a; Sundberg & Porath, 1974; Tomasik & Schilling, 2004) and indirectly (Burton & Harding, 1997b; Lin & Huang, 1992; Slaghek et al., 2001). Direct integration of epoxy groups can be achieved using epichlorohydrin (Burton & Harding, 1997a; Tomasik & Schilling, 2004) or diepoxides (Burton & Harding, 1997a; Sundberg & Porath, 1974). Disadvantages of these routes are the side reactions during the synthesis, use of hazardous reagents like butadiene diepoxide as well as harmful solvents such as dimethyl sulfoxide and dichloromethane. Indirect incorporation of epoxy groups can be accomplished by oxidation of double bonds attached to the polysaccharide using peracids and/or hydrogen peroxide (Burton & Harding, 1997b; Lin & Huang, 1992). Allyl cellulose was efficiently epoxidized using peracids, although this reaction pathway resulted in partial hydrolysis of the cellulose derivative. A major drawback is that the used solvent (dichloromethane) is not applicable for large scale reactions.

In previous studies (Huijbrechts et al., 2007, 2009), double bonds were introduced into starch by the reaction of maize starch and allyl glycidyl ether (AGE). Using mild reaction conditions, starch granules reacted with a small amount of AGE, and low etherified starch derivatives (degree of substitution up to 0.23) were obtained. These low allyl substitutions already induced alteration

Abbreviations: AHP, allylhydroxypropyl groups; AGE, allyl glycidyl ether; AGU, anhydrous glucose unit; Cr_{intra}, cross-links within the fragments; Cr_{inter}, cross-links between two fragments; DL, diol groups; DP, degree of polymerization; DS, degree of substitution; HD, 1,6-hexaandiamide; MG, methyl-α-D-glucopyranoside; *p*-NBP, 4-(*para*-nitrobenzyl)pyridine; OX, epoxy group; WMS, waxy maize starch.

* Corresponding author. Present address: Department of Metabolic and Endocrine Diseases. University Medical Centre Utrecht (UMCU), 3584 EA Utrecht, The Netherlands. Tel.: +31 (0) 88 7554266; fax: +31 (0) 88 7554295.
E-mail address: Annemarie.Huijbrechts@gmail.com (A.M.L. Huijbrechts).

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in properties and behaviour of starch (Huijbrechts et al., 2007, 2008). The current paper presents the results obtained from the epoxidation of double bonds in these allyl waxy maize starch derivatives. Both, the amount of epoxy groups in the product as well as the structure of epoxy starch derivatives were studied. Furthermore, covalent immobilization of an amine and a protein onto the synthesized epoxy starch products was investigated.

2. Experimental part

2.1. Materials

1-Allyloxy-2-hydroxypropyl-waxy maize starch (AHP-WMS; DS_{allyl} = 0.23) was synthesized as described before (Huijbrechts et al., 2008). Acetonitrile (99.5%) and 1,2,5,6-diepoxyhexane (97%) were purchased from Acros Organics (Belgium). Hydrogen peroxide (35% weight solution) was obtained from Chem-Lab NV (Belgium). 1,6-Hexanediamine (HD) (⩾99%) and 4-(*para*-nitrobenzyl)pyridine (*p*-NBP) (⩾98%) were from Fluka (Switzerland). Allyl glycidyl ether (AGE; ⩾99%), 1,3-butadiene diepoxide (97%), 1,2,7,8-diepoxyoctane (97%), potassium carbonate (⩾99%), phosphate salts, dimethyl sulfoxide (DMSO, 99.5%), ethylene glycol (⩾99%), methyl α-D-glucopyranoside (MG), *para*-nitrophenyl-β-D-glucopyranoside (⩾99%), α-amylase (EC 3.2.1.1) (from *Bacillus licheniformis*, 408 U mg^{-1}) and amyloglucosidase (EC 3.2.1.3) (from *Rhizopus* sp., 11600 U g^{-1}) were obtained from Sigma–Aldrich Chemie BV. Pullulanase (EC 3.2.1.41) (from *B. licheniformis*, 400 U mL^{-1}) was obtained from Megazyme (Ireland). β-Amylase (EC 3.2.1.2) (from barley, 45 U mg^{-1}) was purchased from Merck (Belgium). The thermostable enzyme β-glucosidase from the hyperthermophilic archaea *Pyrococcus furiosus* expressed in *Escherichia coli*, was kindly supplied by the Laboratory of Microbiology of Wageningen University.

15-mL Conical centrifuge tubes (PP) were obtained from Corning Incorporated (United States). 3.5-mL Green capped tubes (PP) were purchased from Greiner Bio-One (Germany). Cuvets (Hellma; 1 mm, 45 × 12.5 × 3.5 mm, 350 μL) were from Elcolab (The Netherlands). Micron Centrifugal Filter Devices with Ultracel YM-10 membrane were purchased from Millipore Corporation (United Kingdom).

2.2. Coupling of methyl α-D-glucopyranoside with diepoxides

Methyl α-D-glucopyranoside (MG) (0.97 g, 5 mmol) was dissolved in 5 mL demineralised water in a 25 mL Teflon-sealed bottle. After addition of NaOH (0.10 g, 2.5 mmol) and Na_2SO_4 (0.36 g, 2.5 mmol), the solution was heated to 45 °C in a shaking water bath. Diepoxide (15 mmol; 1.16 mL 1,3-butadiene epoxide, 1.75 mL 1,2,5,6-diepoxyhexane or 2.14 mL 1,2,7,8-diepoxyoctane) was stepwise and slowly mixed to start the reaction. The reaction was followed by thin layer chromatography (eluent: ethyl acetate). After TLC indicated complete conversion of MG, the mixture was cooled in an ice-bath and neutralized by adding 1.0 M HCl-solution (pH 7). Residual diepoxide was removed by extraction with diethyl ether (3 × 5 mL). The aqueous layer was dried by lyophilisation. The products were analyzed by HPLC, using a Waters Spherisorb S5-amino column (250 × 10 mm). Elution of the sample components was by an ethyl acetate–methanol gradient (increased from 7:93 to 25:75 by elution of 1 mL min^{-1}). For detection, an Alltech Evaporative Light Scattering Detector was used. Increasing diepoxide:MG ratios resulted in an increasing number of components in the product. Subsequent LC–MS analysis additionally showed increasing molecular masses of these product components. However, in all cases it was clear that the products did not contain sugars.

2.3. Epoxidation of allylated starch

AHP-WMS (10.0 g, 10% w/w H_2O, 0.051 mmol anhydrous glucose unit (AGU), DS_{allyl} = 0.23) was suspended in an aqueous solution of demineralised water (35 mL), Na_2CO_3 (1.75 mg, 0.017 mol) and $NaHCO_3$ (350 mg, 4.17 mmol), and CH_3CN (5 mL, 0.11 mol). The suspension was stirred, while being heated to 30 °C. The reaction was initiated by adding H_2O_2 (35% wt., 9.4 mL, 0.11 mol) in portions of 100 μL during 30 min. After that, the mixture was stirred at 30 °C for 6 h, then cooled down to room temperature, and finally water (25 mL) was added. The product was obtained by filtration on a glass filter (G3), and subsequently washed with water (5 × 75 mL), ethanol (3 × 75 mL) and acetone (3 × 75 mL). The remaining white powder, epoxy-WMS (9.52 g, 9.5% w/w H_2O, yield: 96%), was dried overnight at room temperature and stored at −20 °C.

2.4. Coupling of 1,6-hexanediamine to epoxy-starch

Epoxy-WMS (100 mg, 1 equiv.) was suspended in water (0.9 mL), followed by addition of 1 mL of 66 mM 1,6-hexanediamine (HD, 7.67 g mL^{-1} in water) corresponding to five equivalents. The suspension was stirred for one night at 30 °C. The product was isolated at room temperature using an Ultracel YM-10 membrane by centrifugation at 13,400 rpm for 1 h and washing with water (5 × 1 mL). After lyophilisation, a white powder was obtained (HD-WMS; 98 mg, yield: 98%).

2.5. Enzymatic degradation

For the enzymatic digestion of the samples, α-amylase, pullulanase, amyloglucosidase and β-amylase were dissolved in deionised water purified by Millipore Milli-Q Gradient A10 (Millipore, United Kingdom), resulting in stock solutions containing 0.44, 0.40, 0.14 and 0.45 U $μL^{-1}$, respectively.

The procedure for the enzymatic degradation of native starch and its derivatives was as follows: Samples (5 mg, 0.3 mmol) were dissolved in 1 mL 0.05 M sodium acetate buffer (pH 5.0). After pregelatinized of the starch solutions for 10 min at 95 °C, incubation was subsequently performed with a combination of pullulanase (5 μL), α-amylase (5 μL) and amyloglucosidase (5 μL) at 40 °C for 18 h. Incubation β-amylase (5 μL) was performed separately at 25 °C for 18 h. Afterwards solutions were boiled for 10 min.

2.6. High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) was carried out using an HPLC (ThermoFinnigan, United States), with three TSK-gel columns in series (7.5 × 300 mm per column, G4000PWXL, G3000 PWXL and G2500PWXL; TosoHaas, Japan), in combination with a PWXL-guard column (40 × 6 mm; TosoH, Japan). Elution was performed at 30 °C with 0.2 M $NaNO_3$ at a flow rate of 0.8 mL min^{-1}. After injection of a 100-μL sample, the eluting compounds were analyzed with a refractive index detector (Shodex RI-101, Showa Denko K.K., Japan). The data were processed using Chromeleon software (Dionex, United States).

2.7. High-performance anion exchange chromatography

High-performance anion exchange chromatography (HPAEC) was performed on an HPLC system (Dionex, United States). The system was equipped with a quaternary gradient pump, an AS3000 autosampler with a helium degassing unit and an ED40 EC detector in pulsed amperometric detection (PAD) mode. The CarboPac PA1 column (2 × 250 mm; Dionex, United States) with a CarboPac PA1 guard column (2 × 50 mm; Dionex) was operated

at a flow rate of 0.3 mL min^{-1} at 20 °C. 20 μL of 10-fold diluted sample was injected and a linear gradient from 0.00 to 0.5 M NaOAc in 0.1 M NaOH within 40 min was applied, followed by a linear gradient in 5 min to 1 M NaOAc in 0.1 M NaOH. Finally, the column was washed for 5 min with 1 M NaOAc in 0.1 M NaOH, and equilibrated for 20 min with 0.1 M NaOH. The data were processed using Chromeleon software (Dionex, United States).

2.8. MALDI-TOF mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) was carried out using an Ultraflex workstation (Bruker Daltonics GmbH, Germany) equipped with a 337 nm nitrogen laser. The mass spectrometer was operated in the positive mode and was calibrated with a mixture of maltodextrins (mass range = 400–3500 Da). After a delayed extraction of 120 ns, the ions were accelerated with a kinetic energy of 25 kV. Hereafter, the ions were detected in the reflector mode. 1-μL 10-fold diluted sample and 1 μL of the matrix solution was mixed and dried on target. The lowest laser power required to obtain good spectra was used. The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in a 1-mL mixture of acetonitrile:water (300 μL:700 μL).

2.9. Determination of epoxy groups

The amount of epoxy groups was measured using a spectrophotometric assay adapted from the literature (Cedrone, Bhatnagar, & Baratti, 2005; Zocher, Enzelberger, Bornscheuer, Hauer, & Schmid, 1999). All assays were performed in tubes (3.5 or 15 mL). Absorptions at 600 nm were measured using 1-mm cuvets in a Cary 100 UV–Vis spectrophotometer, thermostated at 20 °C, and followed with Cary WinUV Kinetic Software. Epoxy-WMS, AHP-WMS as a control, and a calibration curve of 20–100 mM *p*-NBP were used in the two step spectrophotometric assay. The principle of the assay is described in Scheme 1.

2.9.1. Reaction 1

In a 15-mL tube, epoxy-WMS (0.1–0.3 g, 9.5% H_2O, 0.35–0.70 mmol AGU) was suspended in 2 mL of 90 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7, containing 10% v/v DMSO. Subsequently, 2 mL of freshly made 50 mM *p*-NBP (100 μmol) in an ethylene glycol/ethanol mixture (80/20 v/v) was added to the suspension. After vortexing, the tube was placed in a heating bath at 80 °C for 2.5 h. The reaction mixture was cooled on ice for 10 min and centrifuged for 15 min at 15,200 rpm.

2.9.2. Reaction 2

The supernatant was analyzed for the amount of remaining (unreacted) *p*-NBP as follows. 1 mL of the supernatant was pipetted into a 3.5-mL tube and mixed with 1 mL freshly made 50 mM AGE (50 μmol) in 90 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7, containing 10% v/v DMSO. Again, the reaction was performed in a heating bath at 80 °C for 2.5 h. The samples were cooled on ice for 10 min and diluted fourfold. Before blue colour development was induced with 100 μL 1 M K_2CO_3, the diluted mixture (200 μL) was mixed with 200 μL ethanol. The sample was rapidly mixed with K_2CO_3 and the absorption at 600 nm (A_{600}) was measured after 1 min.

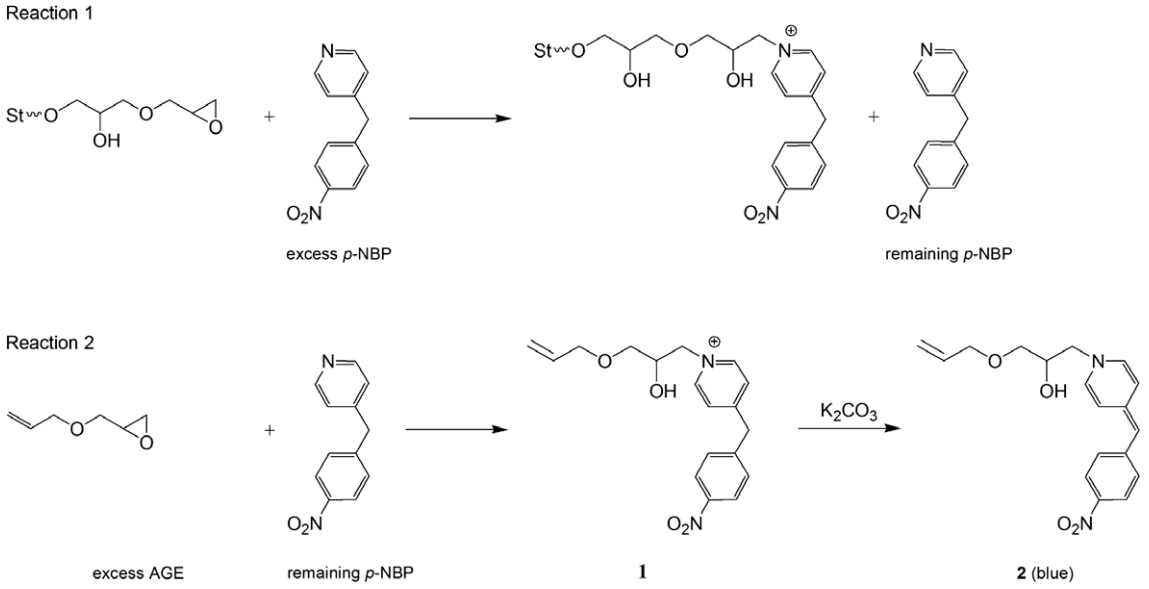
All assays were performed in triplicate, after which the epoxy content was calculated using the calibration curve and the following equation:

$$n_{epoxy} = n_{p\text{-NBP}} - n_2 \quad (1)$$

where n_{epoxy}, $n_{p\text{-NBP}}$ and n_2 are the moles of epoxy groups on the modified starch, the number of moles of *p*-NBP initially (Scheme 1, reaction 1) added and the number of moles of the blue chromophore **2** of *p*-NBP product **1** (Scheme 1, reaction 2), respectively.

2.10. Amino substitution

The nitrogen (N%) and carbon contents (C%) of HD-WMS and epoxy-WMS were obtained using a Fisons EA1108 elemental analyzer. In the DS_{HD} calculation, we assumed that only one amino group of hexanediamine (HD) binds to an epoxy group, leaving one amino group free. DS_{HD}, expressed as moles amino groups per mole glucose units, was determined using *n*N (N%/MW; N-atom MW = 14.01), *n*C (C%/MW; C-atom MW = 12.01), and the number of atoms in the sample according to the following equation:



Scheme 1. Two step spectrophotometric assay to quantify epoxy groups in modified starch (St). *p*-NBP = 4-(*para*-nitrobenzyl)pyridine; addition of base converts product **1** into the blue chromophore **2**.

$$\frac{nC_{HD}}{nN_{HD}} = \frac{C_{AGU} + C_{AGE} \cdot DS_{allyl} + C_{HD} \cdot DS_{HD}}{N_{HD} \cdot DS_{HD}} \quad (2)$$

where nC_{HD} is the moles of C-atoms in HD-WMS; nN_{HD} is the moles of N-atoms in HD-WMS (nN minus the nN from N-containing compounds of epoxy-WMS proportionally); C_{AGU}, C_{AGE} and C_{HD}-atoms correspond to the number of C-atoms in AGU (in starch), in AGE or HD, respectively; N_{HD} is the number of N-atoms in hexanediamine. DS_{allyl} of AHP-WMS is 0.23.

2.11. Physicochemical properties

The temperature of gelatinization of the modified starches was measured using a Differential Scanning Calorimeter (DSC) (Perkin Elmer DSC-7, Boston, USA). Sample preparation and analysis were performed as described previously (Huijbrechts et al., 2008). The PE Pyris – DSC-7 software was used for data handling. Onset temperature (T_o), peak temperature (T_p), completion temperature (T_c) and enthalpy of gelatinization (ΔH_{gel}) were calculated from the DSC thermogram. ΔH_{gel} was based on the dry material (J g^{-1} dry starch). The swelling power and solubility index of starches was determined using the previously published method (Huijbrechts et al., 2008). The experiment was repeated three times. The mean values are reported, with standard deviation.

2.12. Preparation of immobilized β-glucosidase

For preparation of immobilized β-glucosidase, 50 mg of epoxy-WMS was swollen in 1.0 mL 50 mM sodium citrate buffer pH 5.0 at 80 °C for 1 h. Subsequently, 55 μL β-glucosidase (solution of 0.9 mg/mL enzyme) was added and mixed with swollen starch gel. Incubation at 80 °C was continued for 30 min under frequent mixing. Gel formation was completed by cooling the mixture to room temperature.

Release of unbound β-glucosidase from the starch gel was investigated by repeated washing steps followed by centrifugation. The supernatant obtained was stored for the determination of activity recovery of unbound β-glucosidase. Washing of the starch gel was performed by suspending the gel pellet with 0.5 mL citrate buffer pH 5.0 followed by centrifugation at 12,000 rpm, 4 °C for 10 min. The gel pellet obtained after four washing steps was used for determination of the β-glucosidase activity immobilized in epoxy-WMS gel.

2.13. Assay of β-glucosidase activity

Activities of free and immobilized β-glucosidase were assayed using *para*-nitrophenyl-β-D-glucopyranoside as substrate (Hansson, Kaper, Van der oost, De Vos, & Adlercreutz, 2001). Hydrolysis of *para*-nitrophenyl-β-D-glucopyranoside was followed by spectrophotometric measurements at 50 °C *via* continuous determination of the increase of absorbance at λ = 405 nm due to the liberation of *para*-nitrophenol. The reaction mixture contained 1.0 mL 50 mM sodium citrate buffer pH 5.0, with 5 mM *para*-nitrophenyl substrate (final concentration 4.0 mM), and 0.2 mL free enzyme or starch gel.

One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the liberation of 1.0 μmol of *para*-nitrophenol per minute at 50 °C under these conditions, using a molar extinction coefficient of 290 $M^{-1}cm^{-1}$ at 405 nm for *para*-nitrophenol. Activity recovery was related to the activity of free β-glucosidase, obtained after treatment of the enzyme solution in a similar way as in the immobilization procedure, at 80 °C.

3. Results and discussion

3.1. Direct introduction of epoxide moieties

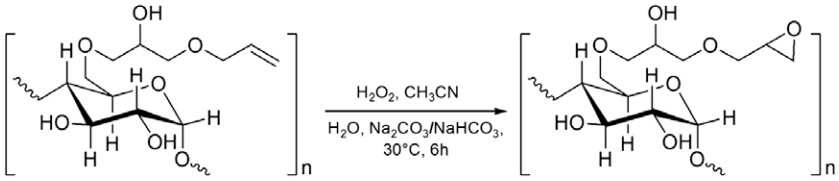
A one-step route to synthesize epoxy starch derivatives is the use of diepoxides (Burton & Harding, 1997a; Sundberg & Porath, 1974). This option was explored in a system consisting of methyl α-glucopyranoside (MG) as a model compound, and 1,3-butadiene diepoxide, 1,2,5,6-diepoxyhexane and 1,2,7,8-diepoxyoctane as epoxide donors. The slightly yellow-coloured clear reaction mixtures were worked up and the products of each reaction were isolated. The transparent foamy products appeared to be a very complex mixture of mainly ring-opened and cyclised diepoxides (Wiggins, 1950) and unreacted MG, as concluded from HPLC and LC–MS analysis (not shown). Reference compounds for HPLC analysis were prepared by reaction of diepoxides under similar conditions, but without MG. Comparing HPLC analysis of products isolated from reactions with and without MG showed that reactions with MG yielded mainly compounds derived from epoxide cross-linking, and unreacted MG (not initially detected by TLC before work-up due to possible entrapment into polymerised epoxides). No epoxy MG derivatives were detected. This indicated that diepoxides rather react with themselves than with another compounds. Therefore, substitution of starch with diepoxides is not an option for the synthesis of epoxy starch derivatives under the conditions applied.

3.2. Epoxidation of allyl starch

A more viable route for synthesizing epoxy starch derivatives is the oxygenation of allylated starch (Slaghek et al., 2001). The double bonds of granular AHP-WMS were epoxidized using H_2O_2 and CH_3CN in slightly alkaline suspension at 30 °C (Scheme 2). The combination of hydrogen peroxide and acetonitrile is a well-known reagent for the epoxidation of carbon double bonds (Chen & Reymond, 1995). A two step colorimetric assay was developed to determine the amount of epoxy groups in the product. The obtained epoxy-WMS was subjected to enzymatic digestion to characterize the structure of the starch derivative.

3.3. Quantitative analysis of epoxy groups

The amount of epoxy groups was determined using a quantitative spectrophotometric assay (Cedrone et al., 2005). In this method, 4-(*para*-nitrobenzyl)pyridine (*p*-NBP) was used to assay epoxides through the formation of a blue chromophore. This assay was tested with epoxy-WMS and *p*-NBP, but the colorimetric analysis of the starch suspension was impossible due to the insoluble nature of the starch. Therefore, a new two step spectrophotometric assay was developed (Scheme 1). In the first step, an excess of *p*-NBP was used to quantitatively convert all epoxide groups on the starch (Scheme 1, reaction 1). A dark blue/green starch derivative was obtained. Subsequently, a small part of the supernatant containing the remaining *p*-NBP was transferred to a tube with a high concentration of allyl glycidyl ether (AGE). In this tube, the remaining *p*-NBP was converted in the *p*-NBP product **1** (Scheme 1, reaction 2). The *p*-NBP product is deprotonated in an alkaline medium using K_2CO_3, resulting in the blue chromophore **2**. The concentration of chromophore **2** was determined by its absorption at 600 nm. The amount of *p*-NBP initially added (reaction 1) minus the amount of **2** formed (reaction 2) equals the amount of epoxy groups on the starch. With this assay, DS_{epoxy} of 0.025 was determined corresponding to 0.13 ± 0.03 mmol epoxy groups per g dry allylated starch (Table 1). This suggests that 11% of the allyl groups were converted into epoxy groups. The controls showed hardly any differences in A_{600}.



Scheme 2. Synthesis of epoxy starch derivatives *via* epoxidation of allylated starch.

Table 1
Substitution levels of epoxy-WMS and hexanediamine (HD) treated epoxy-WMS.

Sample	Epoxy groups (mmol g^{-1})	DS	DS/DS_{allyl} (%)[a]	C (%)[b]	N (%)[b]
Epoxy-WMS	0.13 ± 0.03	0.025[c]	11[a]	43.08	0.24[d]
HD-WMS	n.d.[e]	0.026[b]	11	39.09	0.53

[a] DS_{allyl} = 0.23.
[b] Determined using elemental analysis.
[c] Determined using 4-(*para*-nitrobenzyl)pyridine titration.
[d] Derived from proteins which are present in WMS.
[e] Not determined.

3.4. Amine substitution

Epoxy groups on a surface are able to react with nucleophiles such as NH_2- and OH-groups. Using hexanediamine (HD), the binding of NH_2-groups to epoxy groups was investigated. In this reaction, we assume that one amino group of HD reacts with an epoxy group since there is a 10-fold excess of amino groups. The amount of NH_2-groups in HD-WMS was determined using an elemental analyzer (Table 1). Via (2), a DS_{HD} of 0.026 was obtained. This result corresponds to DS_{epoxy} obtained by the colorimetric assay, which means that every epoxy group in epoxy-WMS has reacted with HD.

3.5. Physicochemical properties

The structural changes upon epoxidation of AHP-starch may affect the physicochemical properties such as gelatinization, swelling and solubility of the granules. Hardly any differences in the gelatinization temperatures between AHP-WMS and epoxy-WMS were observed (Table 2). However, both the swelling power and solubility index decreased after epoxidation of the allyl groups. This suggests that cross-links may have been formed in the starch, although no significant increase of gelatinization temperatures was obtained. In previous research, cross-linked starches exhibited reduced swelling and solubility of the granules, but also increased gelatinization temperatures (Liu, Corke, & Ramsden, 1999; Wang & Wang, 2000).

3.6. Structural characterization of epoxy-starch

The results of the spectrophotometric assay of epoxy groups, and the elemental analysis of the HD reaction, imply that only a small amount of epoxy groups is available for binding to nucleophilic groups. Furthermore, the reduced swelling and solubility of epoxy-WMS suggest that subsequent reactions may have taken place, such as the formation of internal cross-links in the starch granule. To study the structure of epoxy-WMS, the modified starch was enzymatically hydrolyzed. The enzymatically degraded products will give information about the differences in structure between AHP-WMS and epoxy-WMS.

Table 2
Thermal properties,[a] swelling power and solubility index of native starch and its derivatives.

Sample	T_o[a]	T_p[a]	T_c[a]	ΔH_{gel}[a]	Swelling power (g g^{-1})	Solubility index (%)
WMS	57.3	69.4	75.7	13.8	4.5 ± 0.2	0.2 ± 0.0
AHP-WMS	49.1	56.6	65.5	5.5	14.7 ± 1.0	7.8 ± 1.0
Epoxy-WMS	50.3	56.5	64.7	4.9	12.9 ± 0.2	5.4 ± 0.2

[a] Measured by differential scanning calorimetry; T_o, T_p and T_c are the onset, peak and completion temperature, respectively, and ΔH_{gel} is the enthalpy of gelatinization.

3.6.1. Enzymatic digestion with β-amylase

The extent of β-amylase hydrolysis of native starches and its derivatives was studied using HPAEC and HPSEC elution profiles. These profiles showed that hydrolysis of the AHP-WMS and epoxy-WMS liberated less maltose than the native starch hydrolysis (results not shown). Furthermore, the relative amount of liberated maltose was determined at 89% of native release for AHP-WMS and at 73% for epoxy-WMS. This suggests that enzymatic degradation of epoxy-WMS was more sterically hindered due to the presence of intra- or intermolecular ether cross-links. Derivatization, especially at surface, leaves quite some native-like molecules. According to other studies, cross-linked starch derivatives are less accessible for enzymatic hydrolysis than their non-cross-linked precursors (Hood & Mercier, 1978; Wang & Wang, 2000).

3.6.2. Enzymatic digestion with pullulanase, α-amylase and amyloglucosidase

To study the structure of epoxidized WMS in more detail, epoxy-WMS, AHP-WMS and non-modified starch were also subjected to simultaneous enzymatic digestion using pullulanase, α-amylase and amyloglucosidase. HPSEC elution profiles of both WMS derivatives show remaining high molecular weight enzymatic resistant fragments that are not presented in the native starch digest (results not shown), i.e. the enzymes were sterically hindered (Huijbrechts et al., 2007) in both WMS derivatives.

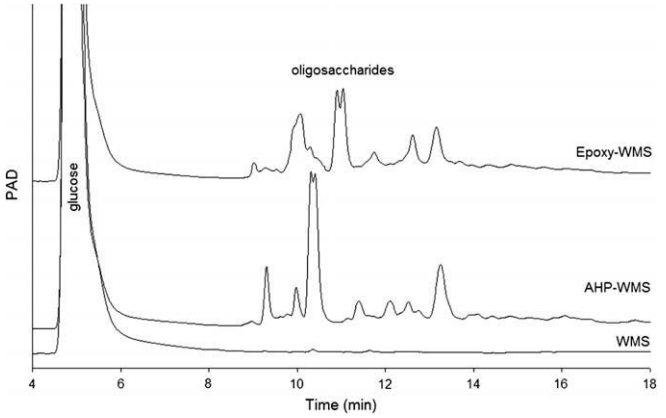


Fig. 1. HPAEC elution profiles of oligomers mixture of WMS, AHP-WMS and epoxy-WMS obtained after enzymatic degradation by pullulanase, α-amylase and amyloglucosidase.

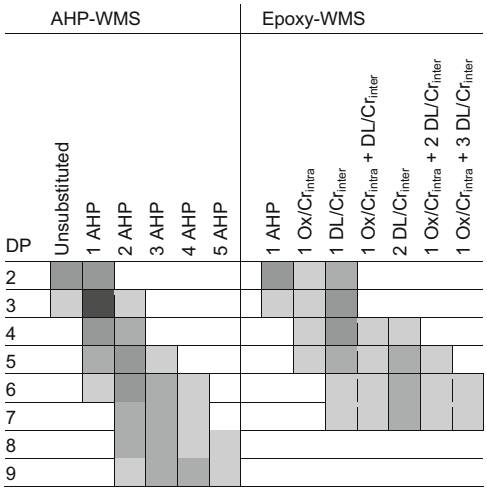


Fig. 2. Mass distribution of the oligomeric fragments of AHP-WMS and epoxy-WMS digests obtained after enzymatic degradation by pullulanase, α-amylase and amyloglucosidase. Enzyme-resistant oligomeric fragments of AHP-WMS contain one to five allylhydroxypropyl groups (AHP), whereas fragments of epoxy-WMS contain AHP or epoxy groups (Ox) as well as diol groups (DL) and cross-links within the oligomer (Cr_{intra}) or between two fragments (Cr_{inter}). DP: degree of polymerization. The total signal intensities of the oligomers in a certain DP are normalized to 100%. Relative intensities 0 100%.

Likewise, the HPAEC elution profiles of both AHP-WMS and epoxy-WMS (Fig. 1) show high molecular weight oligomers which are resistant to further enzymatic digestion. All fragments were eluted within 20 min. Different molecular weight patterns of the enzymatically generated oligomeric fragments were obtained for AHP-WMS and epoxy-WMS. The oligomeric fragments of AHP-WMS eluting at 9.3 min, between 10.3–10.4 and at 12.2 min were not presented in the profile of digested epoxy-WMS, whereas other enzyme-mediated degradation products appeared at 10.1, 10.8–11.4 and 12.7 min.

The differences in the oligomeric fragments released from epoxy-WMS and AHP-WMS after enzymatic digestion was visualized in more detail by MALDI-TOF MS (Fig. 2). In the MALDI-TOF mass spectrum of AHP-WMS digest, enzyme-resistant substituted oligosaccharides with various degrees of polymerization (DP) were identified, ranging from two to nine DP with one to five allylhydroxypropyl groups (AHP). The mass spectrum of epoxy-WMS showed a regular pattern with more and diverse oligomers (DP ranging from two to seven). The mass distribution of the epoxy-WMS digest contained several different enzyme-resistant oligomers having epoxy groups (Ox) and unreacted AHP groups. In addition, oligomeric fragments containing diol groups (DL) were found. Furthermore, there were fragments possessing internal cross-links (Cr_{intra}) or cross-links between two different fragments (Cr_{inter}). These oligomers with diol groups and cross-links were a result of subsequent reactions of epoxy groups, such as hydrolysis of the epoxy moieties or the formation of intra- and intermolecular bridges with free hydroxyl groups in starch.

Some enzyme-resistant oligomeric DP3 fragments that can theoretically be obtained after enzymatic hydrolysis of epoxy-WMS are illustrated in Fig. 3. Fragments containing epoxy groups (Ox) and cross-links within the oligomer (Cr_{intra}) have the same mass over charge ratios, as is illustrated in Fig. 3. Thus, they are indistinguishable in the MS analysis. Similarly, the oligomers having diol groups (DL) and cross-links between two fragments (Cr_{inter}) give the same mass over charge ratios in the mass spectrum of epoxy-WMS. The diversity of possible enzymatically degraded fragments becomes larger for oligomers containing more than three glucose units. The fragments at higher *m*/*z* values (Fig. 2)

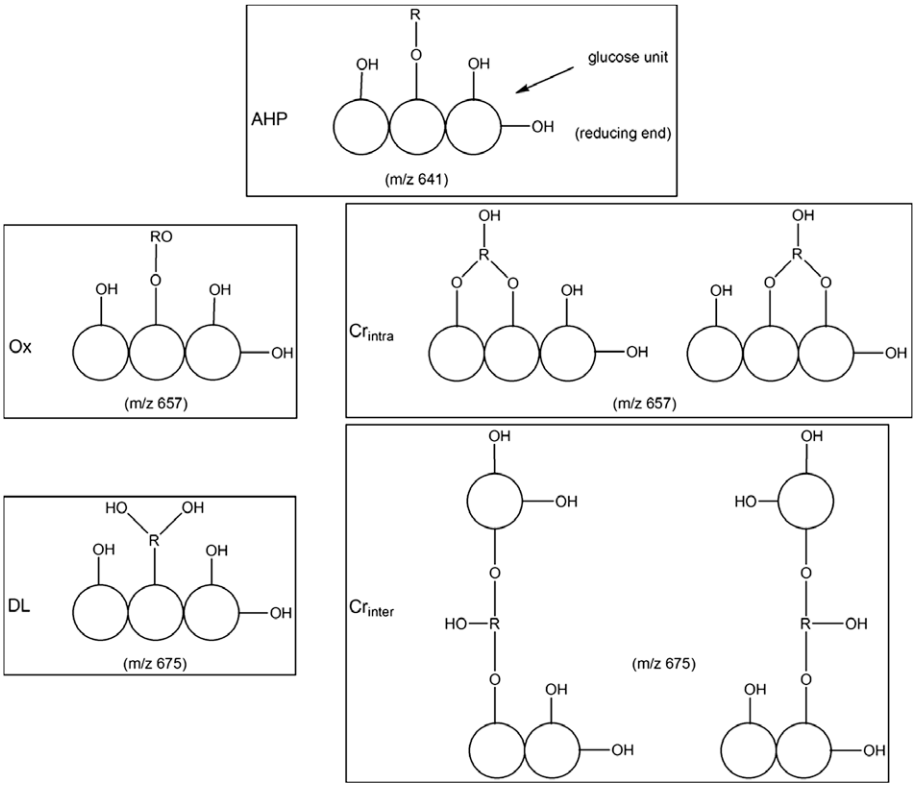


Fig. 3. Simplified scheme of some possible different enzyme-resistant DP3 fragments of the pullulanase, α-amylase and amyloglucosidase digest of epoxy-WMS. Substituted DP3 fragments may contain one allylhydroxypropyl group (AHP, *m*/*z* 641), an epoxy group (Ox, *m*/*z* 657), a diol group (DL, *m*/*z* 675), a cross-link within the oligomer (Cr_{intra}, *m*/*z* 657) or a cross-link between two fragments (Cr_{inter}, *m*/*z* 675). For reasons of clarity, only fragments with a substituent at the central glucose unit are drawn. DP: degree of polymerization; R: AHP group; RO: epoxidized AHP group.

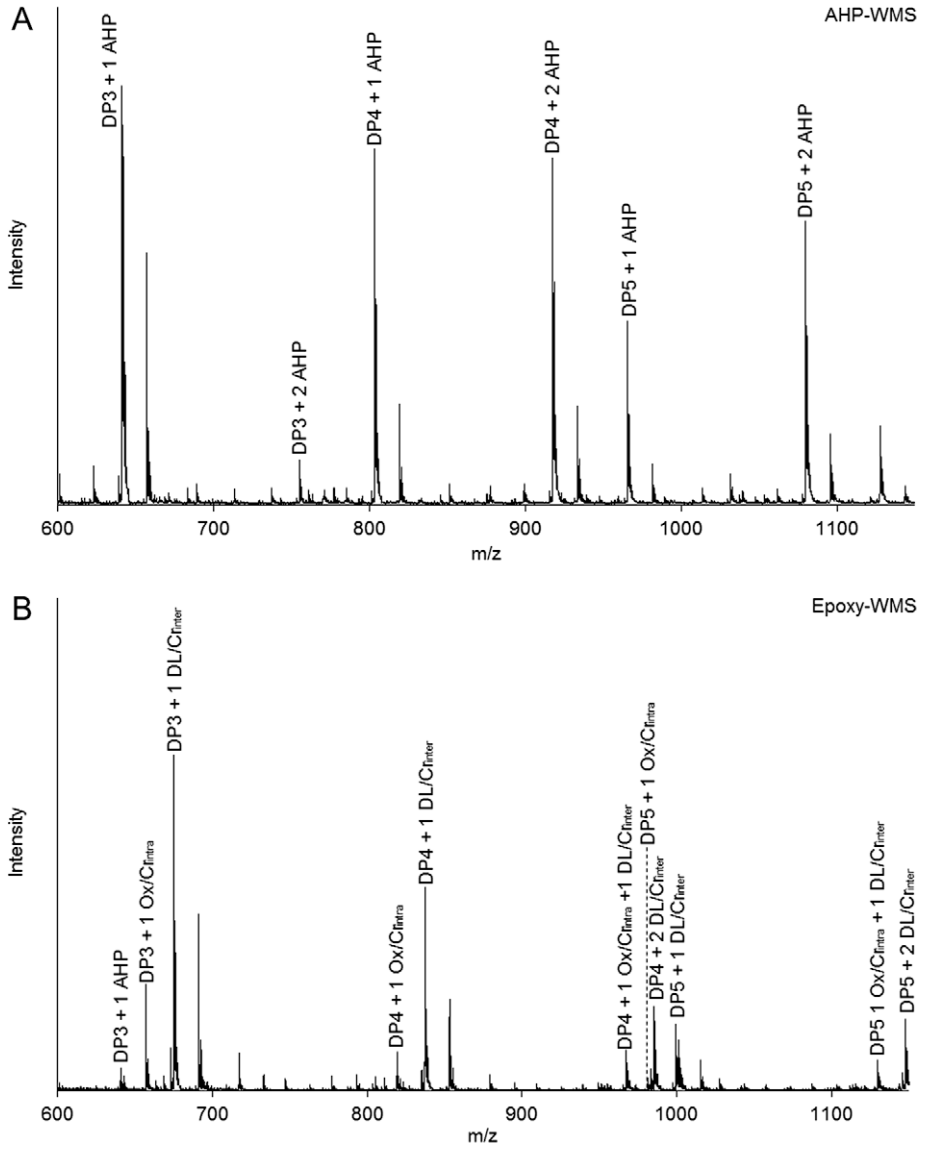


Fig. 4. MALDI-TOF mass spectra ranging from *m*/*z* 1600 to 2000 of the pullulanase, α-amylase and amyloglucosidase digests of AHP-WMS (A) and epoxy-WMS (B). Enzyme-resistant oligomeric fragments of AHP-WMS contain allylhydroxypropyl groups (AHP), whereas fragments of epoxy-WMS contain AHP or epoxy groups (Ox) as well as cross-links within the oligomer (Cr_{intra}) and between two fragments (Cr_{inter}), or diol groups (DL). DP: degree of polymerization.

Table 3
Enzymatic activity recovery of β-glucosidase by immobilization in pregelatinized epoxy-WMS.

Enzyme localization	Enzymatic activity (U)[a]	Recovery (%)
Free β-glucosidase	25.0 ± 0.5	107
Free β-glucosidase, under immobilization conditions[b]	23.0 ± 0.4	100
Immobilized β-glucosidase[c]	2.9 ± 0.5	13
Washed from the starch gel 1st step[d]	15.4 ± 0.6	67
Washed from the starch gel 2nd step[d]	2.7 ± 0.6	12
Washed from the starch gel 3rd step[d]	0.83 ± 0.6	3.6
Washed from the starch gel 4th step[d]	0.16 ± 0.01	0.7

[a] Measured in an enzymatic activity assay for β-glucosidase.
[b] Immobilization conditions: incubation of β-glucosidase (0.05 mg, 0.9 mg mL^{-1}) in 1 mL citrate buffer pH 5.0 at 80 °C for 30 min.
[c] Immobilization of 0.05 mg β-glucosidase in 50 mg pregelatinized epoxy-WMS in 1 mL citrate buffer.
[d] Enzymatic activity of β-glucosidase in the supernatant after washing the epoxy starch gel.

could not be unambiguously assigned due to their large diversity, low relative intensities and overlap of signals.

In the enlargements of the MALDI-TOF mass spectra (Fig. 4), differences in the oligomeric structure and distribution after enzymatic degradation of AHP-WMS and epoxy-WMS, respectively, are clearly shown. The maltriose containing one AHP group is still present after epoxidation. Next to this oligomer, its epoxidized fragment or trioligosaccharide with Cr_{intra} is identified at the signal of 657 *m*/*z*, followed by DP3 with Cr_{inter} or DL (*m*/*z* 675). These differences in oligomeric fragments are also observed for fragments containing four or five glucose residues. Moreover, tetra- and pentaoligosaccharide with two Cr_{inter} or DL, and with one epoxy group or Cr_{intra}, and one cross-link between two fragments or a diol group are found for epoxy-WMS. Furthermore, DP5 with one Ox group or Cr_{intra} and two Cr_{inter} or DL are identified in the MS analysis.

These results show significant differences in the oligomeric fragment patterns of AHP-WMS and epoxy-WMS after enzymatic degradation. Oligomeric fragments resistant to further enzymatic digestion with several allyl groups were obtained for AHP-WMS,

whereas a regular pattern of more and diverse oligomers was found for epoxy-WMS. A small number of oligomers carrying unmodified AHP groups and epoxy groups were found in the MALDI-TOF MS of epoxy-WMS. Most fragments were identified as fragments decorated with diol groups and/or with ether cross-links generated, respectively, by hydrolysis of the epoxy moieties or by the formation of covalent bonds within the oligomer or between two different fragments. These subsequent fragments may have been formed during the epoxidation or storage of the compound, but it is also possible that these fragments were generated during the enzymatic digestion. This suggests that a larger amount of allyl groups in AHP-WMS was converted into epoxy groups than determined with *p*-NBP test, but a significant amount of the epoxy groups reacted further with nucleophilic OH-groups in glucose units or water to generate cross-links or diol groups, respectively.

3.7. Enzyme immobilization in epoxy starch gel

A small amount of epoxy groups in the synthesized epoxy starch derivatives is available for covalent binding with biomolecules. Therefore, epoxy-WMS was employed as carrier matrix for entrapment of the thermostable β-glucosidase from *Pyrococcus furiosis*. The immobilization of β-glucosidase was most efficiently done during the swelling process of the epoxy starch derivative. After pregelatinization of the epoxy-WMS, β-glucosidase was immobilized by mixing the thermostable enzyme with the swollen starch gel. The enzymatic activity of covalently immobilized β-glucosidase was determined by the activity recovery assays of β-glucosidase after washing the carrier matrix several times (Table 3). The enzymatic activity in the epoxy starch gel and the supernatant of the washing steps was compared with the activity recovery of free β-glucosidase under the immobilization conditions. The immobilization procedure is very mild, since free enzyme has still 92% activity under the conditions used during the immobilization. However, most of the enzyme appears to be loosely bound, since only 13% of immobilized enzyme activity remains after four rounds of washing. The total recovered activity of the immobilized enzyme plus the washings is 96%, again showing the mildness of the procedure. To conclude, β-glucosidase was predominantly immobilized onto epoxy starch by weak adsorption. A small part of the enzyme molecules are firmly bound by covalent linkages. The latter is undoubtedly due to the epoxy groups in the starch matrix.

4. Conclusions

We have studied two ways to make epoxy derivatives of (poly)saccharides. The first method involved direct coupling of a sugar derivative to diepoxides. This approach did not lead to the desired products, since only epoxide-derived cyclisation products were recovered. Much better results were obtained with an indirect approach, in which allylated starch was epoxidized using hydrogen peroxide and acetonitrile. A two step spectrophotometric assay was developed to determine the amount of epoxy groups in starch. Subsequently, a DS_{epoxy} of 0.025 was determined corresponding to a quantity of epoxy groups of 0.13 mmol g^{-1} per dry allylated starch. Epoxy starch derivatives exhibited reduced enzymatic susceptibility, swelling capacity and solubility compared with AHP-WMS. Enzymatic hydrolysis elucidated that a larger amount of allyl groups of AHP-WMS was converted into epoxy groups than determined in the colorimetric assay. However, only a small amount of epoxidized fragments were found, due to secondary reactions of the reactive epoxy groups, like intra- and intermolecular cross-linking with free hydroxyl groups of polysaccharide chains, and hydrolysis. In addition, epoxy starch derivatives were employed as carrier matrix for a biomolecule, more particular, enzyme. β-Glucosidase was covalently immobilized to pregelatinized starch, 13% enzymatic activity remained after extensive washing of the epoxy starch gel. This study provides a method to functionalize nongelatinized starch with a reactive group suitable for the development of delivery systems and as carrier for entrapment of biomolecules.

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