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New methods to determine the extent of reaction of epichlorohydrin with maltodextrins

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Abstract

The reaction of epichlorohydrin with maltodextrins has been studied using novel methods for determining free glycerol and glycerol monoethers. Glycerol is determined by enzymatic analysis and glycerol monoethers by a modified oxidative method involving the HPLC separation and the determination of formaldehyde as the 2,4 dinitrophenylhydrazone derivative. Chloride is determined by a standard gravimetric assay involving silver precipitation. These methods permit the deduction of the overall yield of grafting and the degree of cross-linking in the crude reaction medium with no need for further purification thus allowing the study of the reaction of epichlorohydrin with polysaccharides other than high molecular weight starch or dextrans. Other reaction intermediates such as chloropropanediol and glycidol can also be concluded to be produced, based on the kinetics of the hydrolysis reaction.

Keywords: Epichlorohydrin; Maltodextrin; Cross-linking; Analysis

1. Introduction

Epichlorohydrin (ECH) is widely used for the cross-linking of carbohydrates for use in applications ranging from chromatographic supports to medical applications such as wound dressings, biodegradable starch microspheres or polymeric nanoparticles [1–5].

The reaction of ECH with starch and the quantitative evaluation of its cross-linking ability has already been reported [6–9]. During the cross-linking reaction, unreacted ECH undergoes hydrolysis to give glycerol. Glycerol is typically separated by filtration from the polysaccharide network and is then further oxidized with sodium periodate to give two formaldehyde molecules, which can be quantitated by spectrophotometric methods based on the chromotropic acid reaction [6]. In a second step, the purified cross-linked polysaccharide is subjected to periodate oxidation and the formaldehyde derived from the oxidation of glycerol monoethers is separated from the gel and titrated by the same methods.

This methodology is valid for ECH cross-linked starch; the data are more problematic for the case of ECH cross-linked water-soluble carbohydrates, particularly low-molecular-weight oligosaccharides, since the unreacted glycerol cannot be eliminated by simple filtration and the colorimetric method generally used interferes with other aldehydes resulting from sugar oxidation. Moreover the differentiation between free glycerol and glycerol monoethers becomes nearly impossible.

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In this report, a novel method is described for the determination of free glycerol and monoglycerol ethers resulting from the reaction of ECH with low-molecular-weight maltodextrins. The methodology can be applied to the starting reaction medium with no further purification being necessary.

2. Experimental

Reagents.—Polysaccharide hydrolysates (maltodextrins) from maize starch type Glucidex 6 (dextrose equivalent 5%, average M_w: 3200 Daltons) were obtained from Roquette Freres (Lestrem, France).

Epichlorohydrin (1-chloro-2,3-epoxypropane), sodium hydroxide, hydrochloric acid, nitric acid, acetic acid, sulfuric acid, formaldehyde, sodium periodate, sodium borohydride, silver nitrate, lead nitrate and 2,4-dinitrophenylhydrazine were purchased from Fluka (Buchs, Switzerland). Ethanol and acetonitrile were obtained from Carlo–Erba (Nanterre, France).

A glycerol enzymatic assay kit 'Glycerol in food additives', obtained from Boehringer Mannheim (Meylan, France), was used for the analysis of glycerol.

Materials.—All reactions were carried out in a double-jacketed 500 mL glass reactor equipped with a thermostatic bath (RTE 140, Neslab, Paris, France) with magnetic stirring (Heidolph MR 3000, Bioblock, Illkirch, France). Spectrophotometric determinations were performed on a Perkin–Elmer Lambda 12 UV–vis spectrophotometer (Perkin–Elmer, Norwalk, USA). Centrifugation was carried out on a Sigma 3 k 20 Centrifuge (Bioblock, Illkirch, France). Formaldehyde derivatives were analyzed using an HPLC system equipped with a Perkin–Elmer 250 binary pump, an Automatic Sampler ISS 2000, an LC 90 BIO UV detector, a PE Nelson model 10205 Integrator (all from Perkin–Elmer, Norwalk, USA) and a Nucleosil C-18, 5μm, 15 cm HPLC column (Supelco, France).

Epichlorohydrin hydrolysis.—Sodium hydroxide (2 N, 100 mL) was added to a double-jacketed glass reactor and maintained at 20 °C. The desired amount of epichlorohydrin, 2.6 g (280 mmol/L), 0.52 g (56.1 mmol/L) or 0.26 g (28 mmol/L), was added and the reaction was stirred for 12 h. Aliquots were removed at different times and neutralized with either HCl for the determination of free glycerol or with HNO_3 for the determination of free chloride. Samples were analyzed immediately.

Reaction of epichlorohydrin with polysaccharides.—Dry maltodextrin (100 g) was poured into a 500 mL double-jacketed glass reactor and dissolved in sodium hydroxide (2 N, 220 mL) with gentle stirring at 20 °C. Alternatively, 0.5 g of sodium borohydride was added and the reduction reaction was then allowed to proceed for 2 h. Epichlorohydrin [5.7 g; 280 mmol/L, 1 ECH per 10 glucose units (AGU)] was then added and the reaction was maintained with gentle stirring at 20 °C. The reaction was stopped after 6, 12 or 24 h by dilution with 1 L of 0.1 N NaOH and the suspension was maintained at 20 °C overnight. The resulting suspension was then neutralized with HCl (determination of unbound glycerol) and the volume was adjusted to 2.5 L with deionized water, resulting in a maltodextrin concentration of 40 g/L ready for analysis. Maltodextrin controls were prepared in the same alkaline conditions without the addition of ECH.

Determination of unbound glycerol.—Free glycerol was determined by enzymatic assay using the Boehringer specific enzymatic kit; ECH, chloropropanediol or glycidol do not react under these conditions. The kit is based on sequential enzymatic reactions involving NADH. The amount of NADH oxidized is monitored spectrophotometrically at 340 nm. In a typical assay, the suspension (100 μL) is diluted with a ready-to-use buffer solution (1.9 mL) containing NADH and ATP, and then treated with the enzyme preparation (20 μL) for 10 min at 37 °C. The absorbance at 340 nm is read against a blank without glycerol. A calibration curve was prepared using glycerol solutions in the concentration range of 0.5–5 mmol/L.

Determination of free chloride.—Aliquots (20 mL) were removed at different times, neutralized with HNO_3 and $AgNO_3$ was added (80 mL, 0.1 M). The silver chloride precipitate

was filtered through a 0.45 μm glass fiber filter that had been thoroughly dried and accurately weighed. The filter was recovered and dried at 115 °C for 2 h. The amount of chloride liberated in the reaction was determined gravimetrically, based on the weight of silver chloride obtained.

Determination of glycerol monoether by HPLC.—A sample of crude reaction suspension (3 mL), neutralized and diluted as described above, was treated with $NaIO_4$ (3 mL, 0.1 M) for 10 min at 50 °C. After this time, $Pb(NO_3)_2$ (1 M, 1 mL) was added. The white precipitate of lead iodate and lead periodate was removed by centrifugation and the supernatant was recovered (4 mL).

A solution of 2,4-dinitrophenylhydrazine (DPH) was prepared as follows: DPH (400 mg) was dissolved in sulfuric acid (98%, 2 mL) and then diluted to 100 mL with 95% EtOH.

The supernatant from the oxidation process (1 mL) was treated with 1 mL of the DPH solution at room temperature for 1 h. The reaction mixture was then diluted with 95% EtOH (2 mL) and the resulting suspension centrifuged for 10 min at 5000*g* to remove lead sulfate and polysaccharide precipitates. The supernatant was injected directly into the HPLC system.

The HPLC conditions were as follows: column Nucleosil C-18 5 μm, 15 cm; the flow rate was 1mL/min, the mobile phase was 45:55 MeCN–water, the injection volume was 20 μL, and the detection was at 340 nm. Blanks were prepared with a solution of formaldehyde (0–5 mmol/L) treated in the same way as the samples and used to fit the standard calibration curve. Under these conditions the retention time of formaldehyde 2,4-dinitrophenylhydrazone was about 7.7 min ($k' = 4.1$). Quantitative analysis was performed by peak integration using the standard calibration curve.

3. Results and discussion

The reaction of ECH with starch in aqueous alkaline conditions has been extensively studied [6–9]. A classical reaction scheme is shown in Fig. 1. An initial nucleophilic attack of the alcoholate anion takes place to form a monoether of chloropropanediol. The monoether rearranges by chloride displacement to give a new epoxide (Williamson reaction), which reacts with a new alcoholate (Fig. 1(a)) or undergoes alkaline hydrolysis (Fig. 1(b)). In the first case, cross-linking takes place and in the second, a glycerol molecule is grafted onto the polysaccharide network by a monoether bond. Any unreacted ECH hydrolyzes completely to glycerol.

Although this scheme is generally accepted for the reaction of ECH with starch, some reports have suggested considerably more complex structures when ECH reacts with dextrans to give chromatography supports such as Sephadex. In that case, a variety of other structures can be obtained, including ECH dimers and cyclic substituents [10–12].

In the case of starch (or maltodextrins, i.e., α-(1→4) polysaccharides) the C-6 position is free and it is generally accepted that positions C-6 and C-2 are the most reactive hydroxyl groups. In the case of reactions at high base concentration, such as in Williamson reactions, the reactivity ratio is shifted in favour of the C-6 hydroxyl group. Furthermore, cyclizations between C-3 and C-4, as described for dextrans [α-(1→6)-linked polysaccharides], are excluded and cyclizations involving C-2 and C-3 are of low probability. The high ECH concentrations used to obtain dextran gels also explain the formation of dimers and other oligomeric structures.

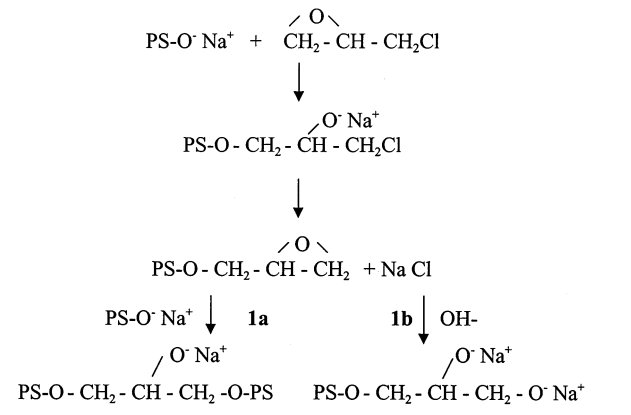

Fig. 1. Proposed mechanism for the reaction of ECH with polysaccharides (PS). (a) Cross-linking. (b) Hydrolysis of the monografted ECH leading to a glycerol monoether.

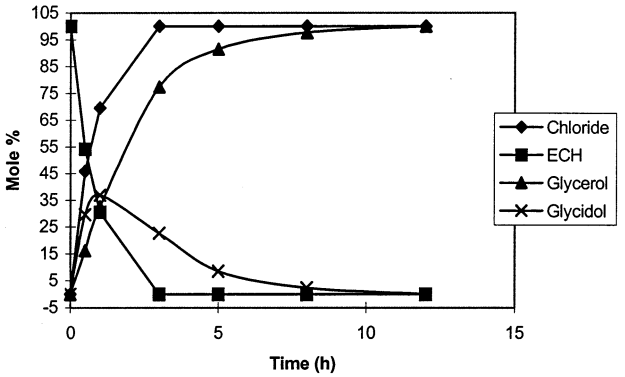

Fig. 2. Hydrolysis kinetics of ECH in alkaline medium (2 N NaOH). Mol% of each reaction product plotted as a function of hydrolysis time.

Cyclic and polymeric constituents of ECH in the reaction with α-(1→4) polysaccharides have been described only when a large excess of ECH is used, for example in cotton fabrics when ECH reacts with cellulose [9]. Considering the conditions used herein, we conclude that the main reactions involve the grafting of glycerol monoethers and cross-linking by a single glycerol diether bond. Further studies would be necessary in order to determine whether other structures are present.

Free glycerol and chloride determinations.— In order to determine the overall yield of the reaction, the usual method is to separate the soluble glycerol from the gel by filtration. The glycerol molecule is then subjected to periodate oxidation, producing two formaldehyde molecules. The resulting formaldehyde is then titrated with chromotropic acid as described elsewhere [13].

In order to avoid the separation of glycerol from the resulting hydrogel, it was titrated using a conventional glycerol enzymatic assay. We first examined the utility of using the enzymatic assay to follow the alkaline degradation of ECH in the reaction medium. The release of chloride was monitored at the same time.

The results (Fig. 2) clearly show that this test is valid for following glycerol formation. We observe that 100% of chloride is released within the first 3 h while 12 h are necessary to obtain 100% of glycerol. This confirms that the first epoxide is rapidly opened and that the Williamsom reaction occurs nearly simultaneously. Neither ECH nor chloropropanediol are present after 3 h of reaction and the hydrolysis mechanism involves the formation of glycidol, carrying the second epoxide, which hydrolyzes slowly (Fig. 2).

We also followed the hydrolysis of ECH at four different concentrations. Fig. 3 clearly shows that, when the concentration of ECH in alkali increases, ECH undergoes autocondensation. The issue of whether bridges with more than one ECH monomer are possible is an open question. However, when ECH reacts with a polysaccharide with molar ratio of 1 ECH/10 AGU, the ratio of polysaccharide hydroxyls to ECH is sufficiently high to conclude that the autocondensation of ECH is

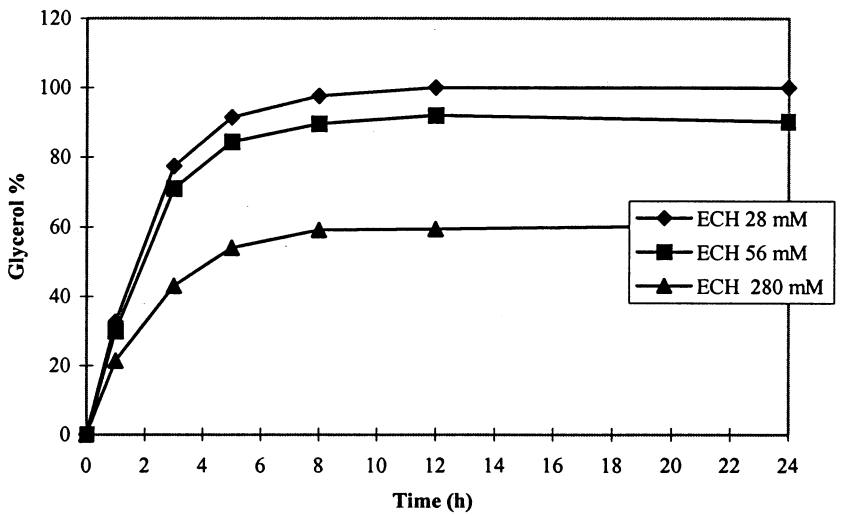

Fig. 3. Kinetics of glycerol formation during ECH hydrolysis at alkaline conditions (2 N NaOH) in the presence of various concentrations of ECH. Glycerol is plotted as the mol% to initial ECH.

Table 1
Glycerol determination by enzymatic analysis during the reaction of ECH with maltodextrins at alkaline conditions [a]

Time (h)	[Glycerol] (mmol/L)	Grafting Yield (%)
6	0.87	96.4
12	0.73	97.0
24	0.63	97.4

[a] Yields are calculated with respect to the initial ECH at 24.4 mmol/L.

negligible. It is clear that as the concentration of ECH increases that possibility becomes higher [9].

The enzymatic assay was also tested for a cross-linking reaction of ECH with a low-molecular-weight polysaccharide, namely a linear starch hydrolysate (maltodextrin) showing a dextrose equivalent of 5%. The average length of the polysaccharide is then about 20 AGU with an average molecular weight of 3200 Daltons.

Glycerol was determined at different reaction times and the results are shown in Table 1. As would be expected from the hydrolysis kinetics, the grafting of ECH on the polysaccharide is quite fast and very high grafting yields are obtained after 6 h of incubation. Nevertheless, a slight increase in the grafting yield can be observed from 6 to 24 h suggesting that the reaction must be run for up to 24 h in order to avoid residual active compounds.

Determination of glycerol monoethers.—The method that we have developed is, like other methods, based on the oxidation of glycerol monoethers and the determination of formaldehyde resulting from the oxidation. The present method differs from other methods in that formaldehyde from the crude reaction medium is determined by reaction with DPH and separation of the corresponding hydrazone by reverse phase HPLC.

Oxidation conditions are described in Section 2 and it has been verified that, under these conditions, the polysaccharide oxidation is complete. Under the same conditions, glycerol gives two molecules of formaldehyde with a yield of 100%. Fig. 4 shows the separation of the formaldehyde 2,4-dinitrophenylhydrazone by HPLC. The three peaks correspond to excess DPH, the formaldehyde derivative and one additional unidentified peak. The linearity of the peak integration is quite good, indicating that the reaction is quantitative for formaldehyde and thus permits the quantitation of formaldehyde in the crude reaction medium.

In order to calculate the degree of glycerol monoether attached to the polysaccharide, different blanks must be taken into account. First, the glycerol molecules obtained from the hydrolysis will give rise to two formaldehyde molecules each. Since glycerol is initially determined via an enzymatic assay, those values are known and can be calculated from the grafting yields shown in Table 1, simply by multiplying the values by two.

Formaldehyde can also be obtained from the oxidation of the polysaccharide itself. We incubated the raw polysaccharide in 2 N sodium hydroxide for 24 h at 20 °C and determined the quantity of formaldehyde obtained after periodate oxidation for each incubation time (Table 2). The results clearly show an increase in formaldehyde content during the incubation.

In fact, the formaldehyde concentration obtained after oxidation of the polysaccharide at zero time (Table 2), is consistent with having been derived from the glucose content in the polysaccharide raw material (0.3% w/w as provided by the supplier), since the periodate

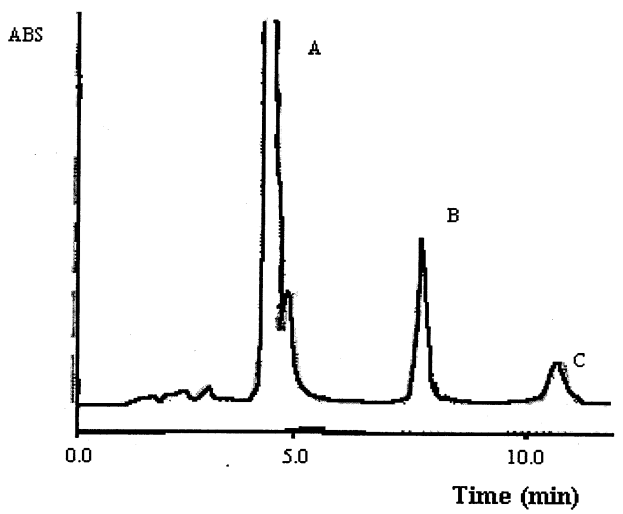

Fig. 4. Chromatographic profile for the determination of formaldehyde by HPLC. Peaks: (A) Unreacted 2,4-dinitrophenylhydrazine; (B) Formaldehyde-2,4-dinitrophenylhydrazone; (C) Unknown. Conditions: Column Zorbax 15 cm × 4.6 mm, flow 1 mL/min; mobile phase 45:55 MeCN/water. Detection 340 nm.

Table 2
Determination of formaldehyde arising from the periodate oxidation of the initial polysaccharide as a function of the incubation time at alkaline conditions (2 N NaOH) [a]

Time (h)	Formaldehyde (μmol/g)	Mol% to AGU
0	16.85	0.27
6	96.55	0.92
12	145.62	1.27
24	234.57	2.03

[a] Formaldehyde is expressed in μmol/g of polysaccharide treated and as the Mol% of degraded AGU to total AGU.

oxidation of glucose gives one formaldehyde molecule.

It is well known [14] that polysaccharides in alkaline conditions suffer a variety of degradation reactions. The degradation proceeds by rearrangement and elimination of saccharinic acids from the reducing end. For maltodextrins, isosaccharinic acids are the main products and these give two formaldehyde molecules on periodate oxidation.

The blank value of formaldehyde in the raw polysaccharide will then be dependent on the incubation time in the alkaline medium.

In view of these results, the quantities of formaldehyde arising from glycerol monoethers in the reacted polysaccharide can be calculated from the following equation:

$$Cmg = Ct - Cg - Cp$$

where: Cmg = formaldehyde concentration from monoglycerol ether; Ct = total formaldehyde concentration determined by the method; Cg = formaldehyde concentration from free glycerol (= enzymatic glycerol, [Glycerol] × 2); Cp = formaldehyde concentration from polysaccharide blank.

The results obtained are shown in Table 3. The overall yield of grafting can be deduced from [Glycerol] and the cross-linking can be calculated from Cmg, both with respect to the total amount of ECH added.

Table 4
Determination of formaldehyde arising from the periodate oxidation of the reduced polysaccharide as a function of the incubation time at alkaline conditions (2 N NaOH) [a]

Time (h)	Formaldehyde (μmol/g)	Mol% to AGU
0	460.17	3.7
6	457.20	3.7
12	460.32	3.7
24	463.50	3.7

[a] Formaldehyde is expressed in μmol/g of polysaccharide and in mol% of reduced glucose to total AGU.

Lastly, it is possible that the blank value of formaldehyde arising from the polysaccharide incubation in alkaline conditions could be affected by the cross-linking process itself. To check this point, a blank polysaccharide was reduced with sodium borohydride and incubated under alkaline conditions. The quantity of formaldehyde arising from periodate oxidation was then determined. As shown in Table 4, the peeling process (alkaline degradation) is eliminated and the formaldehyde value is independent of the incubation time. The reduced polysaccharide was treated with ECH for 12 h and the grafting and cross-linking yield calculated as described above. Table 5 shows the same results for the reduced and non-reduced polysaccharide, indicating that the peeling process is not affected by the cross-linking reaction.

4. Conclusions

The reaction of ECH with a low-molecular-weight model polysaccharide has been investigated using new analytical methods. The overall yield of the grafting of ECH to the

Table 3
Determination of formaldehyde from the ECH treated polysaccharide as a function of reaction time [a]

Time (h)	Cg (mmol/L)	Cp (mmol/L)	Ct (mmol/L)	Cmg (mmol/L)	Cross-linking yield (%)
6	1.74	3.86	9.77	4.17	83
12	1.46	5.82	10.16	2.88	88.2
24	1.26	9.38	12.96	2.32	90.5

[a] Cg is calculated from the enzymatic glycerol [glycerol × 2] from Table 1. Cp is the formaldehyde arising from the raw polysaccharide oxidation. Ct is the total formaldehyde concentration. Cmg is the formaldehyde from glycerol monoethers (Ct − Cp − Cg). The yield of cross-linking is expressed in % to initial ECH.

Table 5
Comparison of grafting yield between reduced and non-reduced maltodextrins treated with ECH under the same conditions (NaOH 2 N, 1 ECH to 10 AGU)

Maltodextrin	Grafting yield (%)	Cross-linking yield (%)
Non-reduced	97.0	88.2
Reduced	96.6	88.9

polysaccharide can be easily calculated by direct titration of glycerol using a classic enzymatic titration kit. The amount of cross-linking can be deduced from the titration of glycerol monoethers in the crude reaction medium. The aldehydes resulting from the periodate oxidation of the cross-linked polysaccharide are readily converted to the 2,4-dinitrophenylhydrazone derivatives. The corresponding formaldehyde derivative can be easily separated by HPLC thus permitting the determination of glycerol monoethers.

The release of chloride is much faster than glycerol formation, indicating a classic Williamson rearrangement mechanism. The reaction is essentially complete in 12 h but 24 h are necessary to ensure the elimination of any residual ECH.

At the conditions used herein, the overall yield of grafting of ECH to polysaccharide is very high ($>95\%$) and more than 85% of ECH molecules are effectively involved in cross-linking.

The methods developed provide a useful tool for studying ECH reactions with any type of carbohydrates, independent of the mechanical characteristics of the resulting hydrogel.

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