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Enzymatic modification of hydroxyethylcellulose by transgalactosylation with β-galactosidases

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Abstract

β-galactosidases from *A. oryzae* and a thermophilic CLONEZYME™ glycosidase library were used to catalyze the transfer of the β-D-galactopyranosyl moiety from lactose to the hydroxyl groups of hydroxyethylcellulose (HEC) in sodium acetate buffer. The degree of substitution was quantified by using galactose oxidase enzymatic assays. Depolymerization was also observed in the course of the transglycosylation reactions. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Galactosyl hydroxyethylcellulose; β-Galactosidases; Transglycosylation; Galactose oxidase; Chemoenzymatic synthesis

1. Introduction

Polysaccharides are a family of complex structures with a high level of diversity. Due to the presence of many reactive hydroxyl groups, polysaccharides present as a difficult class of materials for chemical processing. In order to achieve specificities for the modification of saccharides, selective protecting and deprotecting reactions are generally required. The multiple chemical steps may preclude practical industrial applications. Alternatively, enzymatic reactions are attractive to prepare or modify polysaccharides with high regio- and stereoselectivity [1]. Approaches to the modification of polysaccharides can be mainly classified in the following three categories: depolymerizing, debranching, and synthetic modification. In recent years, enzymatic transglycosylation catalyzed by glycosidases has been the focus of considerable interest [2–6]. These enzymatic syntheses demonstrate transglycosylation ability toward a wide array of acceptors. In an important contribution to enzymatic modification of oligosaccharides, galactosyl and mannosyl cyclodextrins have been prepared through glycosidase-catalyzed transglycosylation reactions [7,8]. However, extension to the modification of polysaccharides using the transglycosylation method has not been well reported [9,10]. In this study, we demonstrate that hydroxyethylcellulose (HEC) derivatives, which are widely used as rheology modifiers, thickening agents, protective colloids, and a variety of other applications, can be transgalactosylated by β-galactosidases using lactose as a donor (Scheme 1).

The enzymes screened for the transglycosylation in this study were from three different mesophilic sources (*A. oryzae*, *B. circulans*, and *E. coli*) and a thermophilic CLONEZYME™ glycosidase library (Di-

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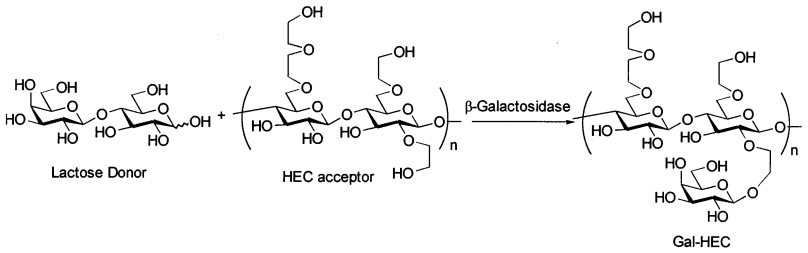


Scheme 1. Transgalactosylation between lactose and HEC.

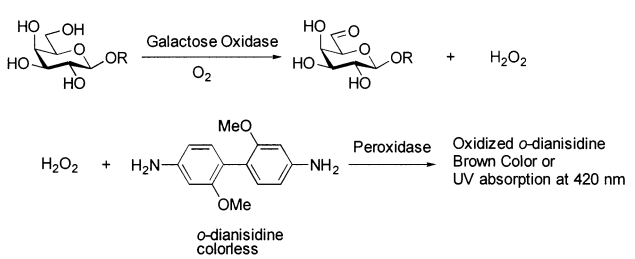
versa). The galactosylated HECs as novel compositions could be useful for hepatocyte attachment in tissue engineering [11].

2. Results and discussion

Since HEC is not a well-defined polymer, the orientations of the hydroxyl groups are randomly distributed along the polymer backbone. Treatment of the enzymes would result in random transgalactosylation. ^{13}C NMR spectroscopy is a conventional means to determine the loci of newly formed glycosidic bonds [12] as well as the degree of substitution on HEC [13]. From the initial study, unfortunately, we found that due to low-efficiency transglycosylation, the spectra of the parent HEC and its galactosylated forms were indistinguishable. Thus, quantification of a small amount of attached D-galactose from the existing glucan backbone chain was carried out by a highly sensitive enzymatic assay that has been used widely in the characterization of D-galactopyranosides in the field of glycobiology [14]. In this enzymatic assay, the galactose oxidase-catalyzed reaction is highly specific for and reportedly more efficient with polymers containing terminal D-galactose. The oxidation reaction can be followed by a peroxidase–chromogen test [15] using a UV spectrophotometer at 420 nm, or it can be observed visually after 60 min of incubation (Scheme 2). Standard curves using β-galactose and lactose both showed excellent linearity between 0 and 278 nmol.

Three different enzymes from *A. oryzae*, *B. circulans*, and *E. coli* were initially screened along with the variation of solvents. The degree of substitution (DS) is defined in terms of each glucosyl unit. The maximum available substitution for each unit is 3. Thus, DS = 3 represents 100% substitution. The results indicated that β-galactosidase from neither *B. circulans* nor *E. coli* generated galactosylated HEC. In the case of *A. oryzae*, the DS of transgalactosylation determined was far less than 0.015 from the peroxidase–chromogen test. In order to reduce the hydrolysis effect, higher concentrations of HEC were used along with an appropriate organic cosolvent such as acetonitrile. However, none of the three enzymes turned out to be better. On the contrary, reduced activity of the enzyme from *A. oryzae* was observed when 50% organic solvent was used.

The effect of reaction time was examined by allowing 2.4 M (82 wt.%) lactose to react with 9 wt.% HEC in sodium acetate buffer (pH 4.85) in the presence of β-galactosidase (10 mg/mL) from *A. oryzae* at ambient temperature. The extent of the transglycosylation was monitored at timed intervals. It is indicated in Fig. 1 that DS was found to reach its maximum after 6 h



Scheme 2. Enzymatic assay via the peroxidase–chromogen test.

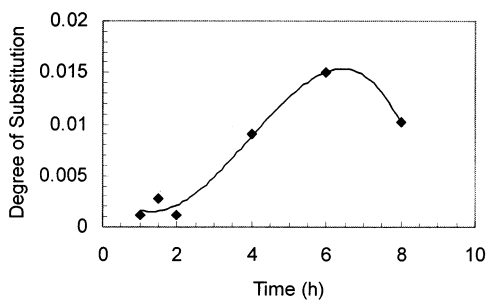


Fig. 1. Gal–HEC observed at timed intervals.

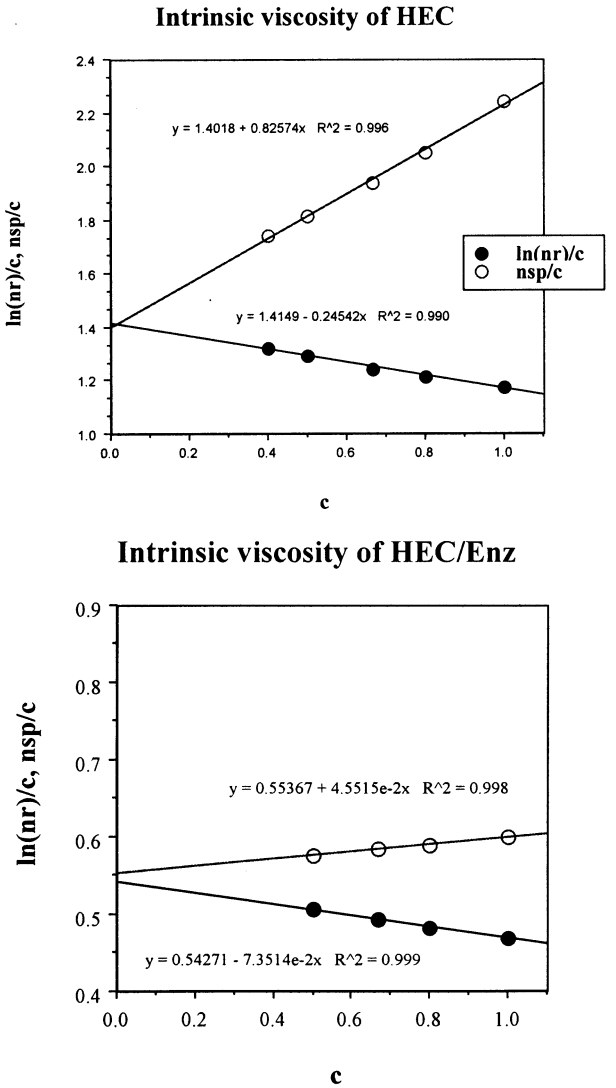


Fig. 2. The relationship of η_r/c and η_{sp}/c toward concentration c in reaction A (HEC only) and B (HEC and enzyme). η_r is relative viscosity and η_{sp} is specific viscosity in which $\lim_{c\to 0}\eta_{sp}/c = \lim_{c\to 0} \ln(\eta_r)/c = [\eta]$.

reaction under the above conditions. Obviously, the optimum DS is no more than 0.015. The ^{13}C NMR spectrum was nearly the same as the one before enzymatic reaction except for the appearance of a pair of α (92 ppm) and β (96 ppm) anomeric signals corresponding to the reducing end of the glucan, which might be the result of the enzymatic depolymerization that occurred during the transglycosylation process.

It is not surprising that commercial β-galactosidase from *A. oryzae* contains other enzymatic activities. The relative activities were found as follows: *o*-nitrophenyl β-galactopyranoside, 100 units; *p*-nitrophenyl β-glucopyranoside, 2.8 units; and *p*-nitrophenyl β-glucopyranoside 1.4 units. The depolymerization could be affected by the weak β-glucosidase activity.

Thin-layer chromatography (TLC) was initially used for the detection of depolymerization. Two reactions were compared: In reaction A, 9 wt.% HEC in sodium acetate buffer at pH 4.5 was used as control, while in reaction B, the same amount of HEC was incubated with the β-galactosidase from *A. oryzae* (10 mg/mL). Both reactions were carried out at ambient temperature for 6 h, and then stopped by boiling for 10 min. From TLC, it was obvious that some degraded products were produced in reaction B besides the existing ethylene glycol oligomers contaminants in the original samples. The corresponding intrinsic viscosity $[\eta]$ was determined by dilute solution viscosity measurement. According to the Kuhn–Mark–Houwink equation, $[\eta] = KM^{\alpha}$, the intrinsic viscosity reflects the magnitude of the average molecular weight of linear polymer [16]. The intrinsic viscosity of HEC obtained in reaction A was 1.4, while the viscosity of HEC obtained in reaction B was ~ 0.55 (Fig. 2). This result demonstrated the depolymerization effect in the transgalactosylation using β-galactosidase from *A. oryzae*.

Effects of different ratios and concentrations of donor and acceptors were studied to search for a better degree of substitution of transgalactosylation reaction. The concentrations and ratios of donor and acceptor used in combination with different enzymes and organic solvents are shown in Table 1.

From these results (Table 1), it is clear that organic solvents debilitate enzyme activities in this reaction, and that CLONEZYME™ thermophilic glycosidase Gly001-02 can catalyze the transgalactosylation reaction. Nevertheless, it did not demonstrate dramatic advantages over conventional enzymes in this reaction. It is noteworthy that a large ratio of lactose donor and HEC acceptor can give a better degree of substitution (up to 0.033) when β-galactosidase from *A. oryzae* was used.

In conclusion, galactosidase-catalyzed transglycosylation can be employed to galact-

osylate water-soluble, cellulose-based polymers. The best enzyme found so far is the conventional β-galactosidase from *A. oryzae*, and the reactions resulted in better yields of Gal–HEC without organic cosolvent. Depolymerization of polysaccharides should not be neglected in the course of the transglycosylation reactions.

3. Experimental

β-Galactosidases (EC 3.2.1.23) from *A. oryzae* and *E. coli* were obtained from Sigma Chemical Co. β-Galactosidase from *B. circulans* was obtained from Daiwa Kasei Co. (Osaka, Japan). Glycosidases of a CLONEZYME™ library were obtained from Diversa. Galactose oxidase and horseradish peroxidase were purchased from Sigma. Chemicals purchased from Aldrich Chemical Co. and Sigma were reagent grade. Natrosol HEC 250LR was provided by Hercules, Inc. The approximate molecular weight is 90,000 Da. It has 400 glucosyl units with each unit having three hydroxyl groups available for glycosylation, regardless of the degree of substitution from ethylene oxide. Analytical TLC was performed on E. Merck plates (Silica Gel F_{254}). Compounds were visualized by staining with a solution of 10% anisaldehyde and 5% H_2SO_4 in methanol, followed by heating on hot plates. Size-exclusion column chromatography was performed using Sepharose CL-4B gel. Dialysis was performed using Spectra/Pro membrane (MW cutoff 8,000 Da).

General method for enzymatic transglycosylation of HEC.—A solution of HEC ($\sim$3 mmol as free hydroxy groups) and lactose ($\sim$0.3 mmol) was prepared at ambient temperature by vigorous agitation in 2.5 mL buffer solution. To the above solution was added a β-galactosidase (2–5 mg) either from a mesophilic source such as *A. oryzae*, *B. circulans* or *E. coli*, or from a thermophilic CLONEZYME™ glycosidase library. The mixture was vigorously agitated for 16 h at ambient temperature, except for the thermophilic enzyme, which was used at 70 °C. The reaction was quenched by heating at 100 °C for either 5 min in the case of mesophilic enzymes or for 25 min in the case of the thermophilic enzyme. The mixture was either directly loaded onto a Sepharose CL-4B gel column and was eluted with water, or it dialyzed exhaustively using Spectra/Pro membrane for 96 h. The resulting polymer solution was determined by TLC on silica gel using 3:7:2 (NH_3–^{i}PrOH–H_2O), followed by lyophilization to afford a white membrane-

Table 1
Transgalactosylation of HEC with different galactosidases

Source	HEC (wt.%)	Lactose (wt.%)	Organic solvent[a]	Reaction time (h)	Degree of substitution[b]
A. oryzae	4	17		6	0.006
A. oryzae	4	34		6	0.012
A. oryzae	4	68		6	0.012
A. oryzae	7	17		6	0.006
A. oryzae	7	34		6	0.012
A. oryzae	7	68		6	0.006
A. oryzae	0.7	34		6	0.024
A. oryzae	0.7	34	Me_2SO	6	0.009
A. oryzae	0.7	34	CH_3CN	6	0.012
A. oryzae	0.7	34		48	0.033
A. oryzae	0.4	34		48	0.030
Gly001-02	9	82		6	0.028
Gly001-02	9	82	CH_3CN	6	0.012
Gly001-02	7	82		48	0.018
Gly001-02	0.7	34		48	0.009

[a] Organic solvents were used at a 1:1 ratio with the aqueous buffer system.
[b] DS values were obtained comparing to a control measurement with HEC only.

like solid. (Buffer solutions with 50 mM and four different pHs were used as follows: sodium acetate buffer pH 4.85 for *A. oryzae*, sodium acetate buffer pH 5.0 for *B. circulans*, sodium phosphate buffer pH 6.0 for Gly001-02 from CLONEZYME™, and sodium phosphate buffer pH 7.0 for *E. coli*.)

Enzymatic assay of glycosidase activities of β-galactosidase from A. oryzae.—To a spectrophotometer cuvette at room temperature was added 1 mL of enzyme solution in sodium acetate buffer at pH 4.5 (2 mg/mL) and 10 μL of 100 mM substrate solution using *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-glucopyranoside, and *o*-nitrophenyl β-galactopyranoside. The amount of *p*-nitrophenolate liberated was measured continuously at 380 nm. One unit of specific activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol *p*-nitrophenolate per minute per mg protein at ambient temperature.

Peroxidase–chromogen test.—The mixed enzyme–chromogen reagent was prepared as follows: 0.5 mL of galactose oxidase (70 units), 0.5 mL of horseradish peroxidase (100 mg/L), 0.5 mL of *o*-tolidine (200 mg/L) and 0.5 mL of the substrate solution (the reaction concentration was less than 1.39×10^{-4} M, i.e., 278 nmol in 2 mL of reaction solution) were mixed and placed in incubator at 30 °C for 1 h. Maximum chromogenesis took place within 60 min. The color developed was read at 420 nm. Due to the complexity of the Gal–HEC, galactose and lactose were both used as standard curves in the range of 278 nmol for the detection of galactose. The Gal–HEC reaction solution absorbance at 420 nm was compared against the standard curve of galactose and lactose.

Intrinsic viscosity.—Viscosity measurements were conducted using an Ubbelohde viscometer. The measurement was carried out using H_2O as the solvent in a constant temperature bath at 25 °C with relative viscosity, $\eta_r = t/t_0$. All flow times were sufficiently long to avoid a kinetic energy correction. The intrinsic viscosity $[\eta]$ was determined as the value of specific viscosity η_{sp}, $(\eta_r - 1)/c$, at the limit of vanishing c.

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