

Chapter 9

Regioselective Enzymatic Transesterification of Polysaccharides in Organic Solvents

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Enzymes catalyze a large number of reactions in non-aqueous media that are not possible in aqueous solution. One of these reactions is the enzyme catalyzed acylation of alcohols. Recently it was observed that enzymes can be extracted from aqueous solutions into organic solvents by using very low surfactant concentrations via ion-pairing between the surfactant and the protein. In the present work we demonstrate the site of selective transesterification of polysaccharides with fatty acids in the presence of ion-paired subtilisin carlsberg. The polysaccharides and corresponding derivatives used include amylose, cyclodextrins, and hydroxyethyl cellulose. Characterization data including FTIR and TGA is reported.

Earlier studies have indicated that subtilisin, a protease, is a useful catalyst in organic solvents to carry out synthetic organic chemistry (1). In organic solvent reaction media this enzyme catalyzes the formation of esters, while in the presence of water, the enzyme hydrolyzes esters (2). Moreover, substrates, such as monosaccharides, used in esterification reactions were soluble in organic solvents, permitting interaction between the soluble substrate and the insoluble enzyme (3). The formation of ion-pairs between an enzyme like subtilisin Carlsberg (from *Bacillus licheniformis*) and a surfactant, was recently studied (4). This catalyst system in organic solvent maintains its activity for the acylation of polysaccharides and oligosaccharides in organic solvents (5). Polysaccharides are of interest because of their biodegradability and biocompatibility properties as well as their wide ranging applications as coatings, finishes, membranes and fibers. Enzyme modified polysaccharides can be useful as biodegradable emulsifiers, compatibilizers, and detergents as well as for surface modification of preformed polysaccharides-based materials (6).

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In this work, we demonstrate that amylose, β -cyclodextrin and hydroxy ethyl cellulose (HEC) (three organic solvent-insoluble polysaccharides consisting of α or β -1,4 linked glucose moieties), when cast as thin films or suspended as cryogenically milled powder, can be transesterified in organic solvents by ion-paired subtilisin protease. Moreover, the results indicated that amylose was regioselectively acylated. These are the first attempts at modifying solvent-insoluble polymers catalyzed by enzymes in organic solvent reaction media.

Materials and Methods.

Subtilisin Carlsberg protease enzyme (Amano Enzyme Co., Troy, VA, 1.1 mg/mL) was dissolved in HEPES buffer (8.5 mM, pH 7.8) containing 6 mM KCl. The aqueous solution was mixed with an equal volume of isooctane containing 2 mM dioctyl sulfosuccinate sodium salt (AOT) and the two-phase solution was stirred at 250 rpm at 25°C. After 30 minutes of stirring, the phases were allowed to settle and the organic phase was removed (5). The protein and water content of the solution were determined by absorbance at 280 nm and Karl-Fischer titration, respectively. Approximately 1.0 mg/mL of enzyme was present in the isooctane solution with a water content of <0.01% (v/v). Amylose was dissolved in water and dried onto ZnSe slides as a thin film approximately 1 μ m in depth. The reactions were performed with 1.0 mg/mL ion-paired subtilisin protease in isooctane containing 60 mM n-capric acid vinyl ester (C10VE, TCI America, Portland, OR) in a beaker containing the ZnSe slide without shaking for 48 h at 37°C. Similar procedures were used for the β -cyclodextrin and for HEC. The solid amylose film was removed from the beaker and washed with fresh isooctane to extract unreacted vinyl ester. Reactions were also conducted with the polysaccharide in powder form, using similar procedures. The polymer powders had a particle size of less than 100 μ m with a surface area to weight ratio of 546 cm²/g.

Results and Discussion

As represented in Fig. 1, the Fourier Transformer Infrared (FTIR) spectrum indicated the formation of a derivatized β -cyclodextrin by the enzyme reaction based on large absorptions at 2920 and 2850 cm⁻¹, corresponding to the methylene stretch of an alkyl chain in the final product. In addition, the presence of a peak in the region 1693-1730 cm⁻¹, indicates a C=O group. Spectra of unmodified β -cyclodextrin or β -cyclodextrin treated with the vinyl ester in the absence of enzyme (Figure 1) do not exhibit these absorptions peaks. Additionally, the lack of absorbance peaks at 871 and 951 cm⁻¹ in the FTIR spectrum indicate the absence of vinyl groups in the modified β -cyclodextrin. Therefore, there was no non-selective adsorption of the vinyl esters to the β -cyclodextrin. Similar results were found for amylose (5) and for HEC.

In addition to the β -cyclodextrin film, cryogenically milled β -cyclodextrin powder was also studied as an isooctane insoluble substrate. The FTIR spectrum of the β -cyclodextrin powder, after the enzymatic reaction, also exhibited evidence of

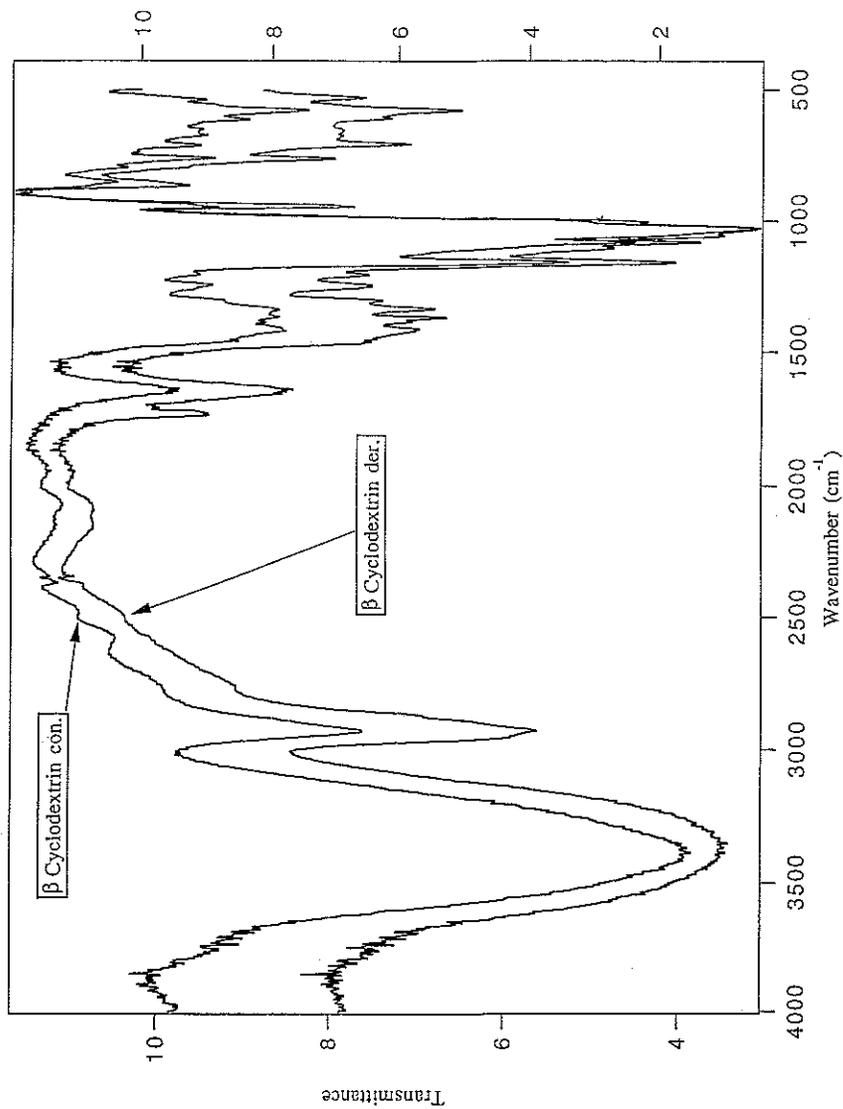


Figure 1. FTIR spectra of unmodified β -cyclodextrin, and modified β -cyclodextrin on ZnSe slides.

acylation analogous to that found in Fig. 1. When subtilisin protease powder, in the absence of AOT (during the extraction procedure), was used as a catalyst, no acylation reaction was observed. Specifically, in the presence of 60 mM vinyl caprate and 10 mg/ml of subtilisin Carlsberg protease powder (without AOT and no ion pairing), no β -cyclodextrin derivatization was apparent when β -cyclodextrin was used either in powder or thin film forms. Consequently, solubilization of the enzyme in an organic solvent in the presence of AOT is important for acylation of the insoluble polymer.

The degree of substitution catalyzed by the enzyme reactions in the powdered β -cyclodextrin and amylose was assessed semiquantitatively by thermogravimetric analysis (TGA) and for amylose also by Electron Spectroscopy Chemical Analysis (ESCA). The TGA profile for native β -cyclodextrin and enzymatically acylated β -cyclodextrin powders are shown in Figure 2. The major difference in the thermograms between the derivatized and unmodified β -cyclodextrin (prior to backbone thermal degradation) was the weight loss in the former at 280°C. This weight loss is characteristic for alkyl chain degradation (7) and was not present in the native β -cyclodextrin. Quantitation of the weight loss of the modified β -cyclodextrin as compared to the unmodified β -cyclodextrin indicates that about 0.18 acyl groups are associated per glucose moiety. Previously, similar results were found for the enzyme-modified amylose acylation by enzyme (6). Chemically and non regioselectively acylated β -cyclodextrin (using acyl chlorides), with higher degree of substitution, shows similar TGA profiles as those reported for the enzymatically-treated polymers (Fig. 2).

These results indicated acylation of surface accessible hydroxyl sites on the polymer chains, a small fraction of the total amylose present in the reaction film. Therefore, ESCA analysis was used to characterize the top 100 Å of the amylose film. This analysis indicated that the acylated surface had a degree of substitution of 0.9 ± 0.1 acyl groups per glucose moiety, based on the C:O ratio. It has been reported that subtilisin protease selectively acylated primary hydroxyl groups in reaction with sugar carried out in organic solvents (8). Therefore, based on the ESCA analysis, it was possible to speculate that regioselective acylation of the primary hydroxyl groups on the polysaccharides also occurred in our procedure. Consequently, $^1\text{H-NMR}$ was used to determine the position of enzymic acylation of amylose. In comparison to underivatized amylose, the only significant shift observed was in the 6-hydroxyl proton of the modified amylose. $^1\text{H-NMR}$ of the enzymatically modified amylose powder and β -cyclodextrin show peaks at 0.8 and 1.2 ppm representing CH_3 and CH_2 protons, respectively, confirming the presence of a straight-chain moiety on the derivatized amylose and β -cyclodextrin. Such groups do not exist in the unmodified polymers.

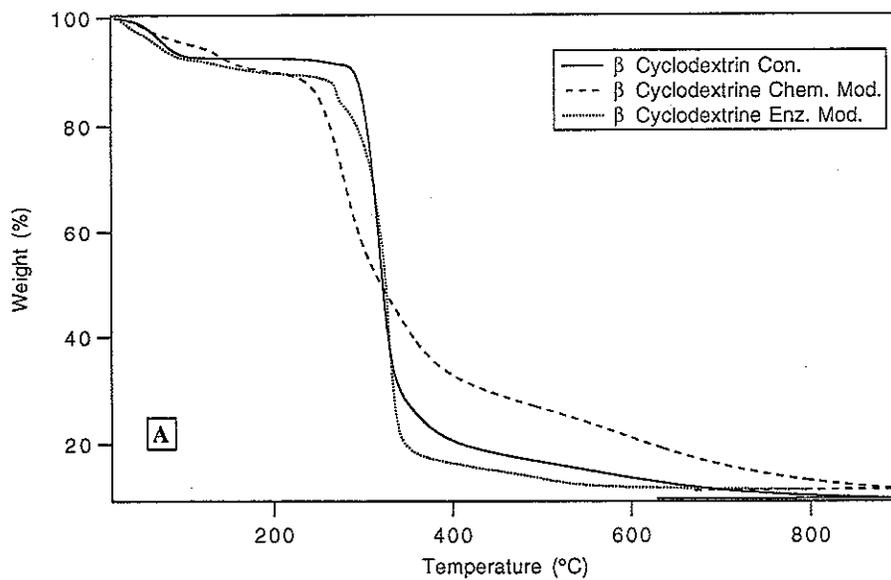
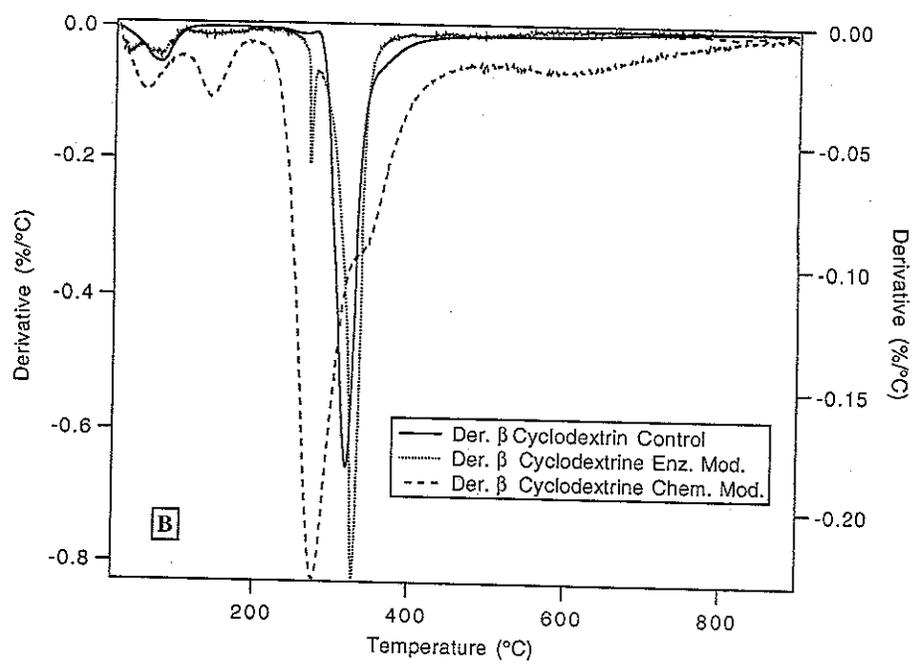


Figure 2. Thermogravimetric analysis of (A) unmodified, enzymatically modified and chemically modified β -cyclodextrin and (B) derivatives (Δ weight/temp.) of unmodified, enzymatically modified and chemically modified β -cyclodextrin.

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Figure 2. *Continued*

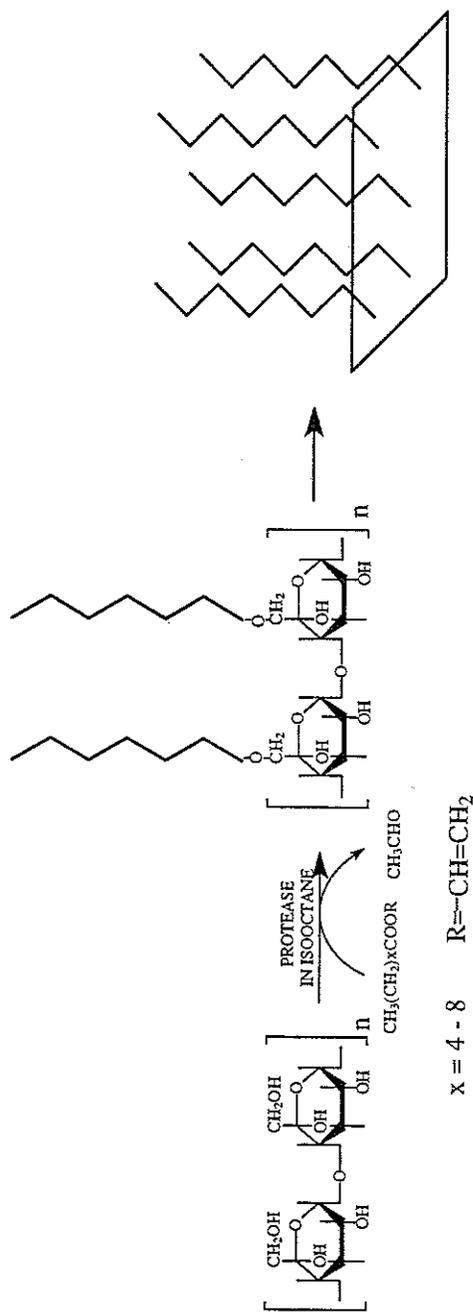


Figure 3. Proposed structure of modified polysaccharides prepared by subtilisin Carlsberg protease catalysis.

Enzymatic specificity of acylation for amylose was demonstrated by $^1\text{H-NMR}$ (*DMSO-d6*): (a) native amylose : δ 3.8 (2 H, 3 H, 4 H, 6 H, br), 4.3 (6 H, m), 4.5 (6 OH, m, area 0.348), 4.9 (1 H, ax, m), 5.2 (1 H, br, area 0.336), 5.5 (3 OH, br, area 0.330), 5.6 (2 OH, br, area 0.330);

(b) derivatized amylose : δ 0.8 (CH_3 , br), 1.2 (CH_2 , br), 1.3 (CH_2 , br), 2.2 (CH_2 , br), 3.75 (2 H, 3 H, 4 H, 6 H, br), 4.4 (6 OH, m, area 0.467), 5.2 (1 H, br, area 0.509), 5.4 (3 OH, br, area 0.495), 5.45 (2 OH, br, area 0.495). It should be noted that the area ratio of the 6-OH proton to total protons in the native and derivatized amylose is 0.26 and 0.23, respectively. This finding provided additional evidence that acylation was confined to the 6-OH group in this films.

Integration of the alkyl chain protons of the amylose derivatives and the β -cyclodextrin protons resulted in a calculated degree of substitution of 0.185 and 0.250, respectively. This is slightly lower than that predicted by TGA analysis of the powdered amylose. Such a discrepancy may result from the relatively qualitative nature of TGA analysis as compared to $^1\text{H-NMR}$.

Figure 3 illustrates the proposed structure of the modified amylose. Subtilisin protease, when surfactant ion paired, appears to be highly efficient in catalyzing the acylation of nearly all available surface primary hydroxyl groups in amylose thin films or fine powder. This may provide a new route to modify surface properties of polysaccharide films or fiber to control hydrophobicity and reactivity.

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