Enzymatic Modification of Insoluble Amylose in Organic Solvents

Ferdinando F. Bruno,[†,‡] Joseph A. Akkara,[‡] Madhu Ayyagari,[‡,§] David L. Kaplan,[*,‡] Richard Gross,[§] Graham Swift,[∥] and Jonathan S. Dordick[*,†]

Department of Chemical and Biochemical Engineering and Center for Biocatalysis and Bioprocessing, University of Iowa, Iowa City, Iowa 52242, Biotechnology Division, U.S. Army Natick Research, Development, & Engineering Center, Natick, Massachusetts 01760, Department of Chemistry, University of Massachusetts at Lowell, Lowell, Massachusetts 01854, and Rohm & Haas Company, 727 Norristown Road, P.O. Box 904, Spring House, Pennsylvania 19477-0904

Received August 23, 1995
Revised Manuscript Received October 19, 1995

Enzymes are powerful catalysts in organic solvents where they catalyze a wide variety of reactions that are difficult to perform in aqueous solutions.[1] This is particularly evident in transesterification reactions catalyzed by lipases and proteases wherein a variety of nucleophiles act as substrates for enzyme-catalyzed acyl transfer in nearly anhydrous organic solvents.[2] In the vast majority of cases, these substrates are hydrophobic and highly soluble in a great number of organic solvents. Thus, adequate interaction between the soluble substrate and the insoluble enzyme[3] is achieved and catalysis ensues. Unfortunately, many polyhydroxylated compounds are either sparingly soluble in only the most polar organic solvents or are completely insoluble in organic media. For these substrates, conventional nonaqueous enzymology is unable to support catalytic transformations, although the high selectivity of enzymes would be a distinct advantage over relatively nonselective chemical approaches. A case in point is the regioselective acylation of polysaccharides to give amphiphilic, biodegradable/biocompatible materials that can be used in injection molding operations, biodegradable emulsifiers, compatibilizers, and detergents.[4] The development of a suitable technique for the selective modification of polysaccharides in organic solvents, therefore, represents both an opportunity for the synthesis of novel materials and a means to overcome a technical hurdle in the broader uses of enzymes in nonaqueous media.

The problem of employing insoluble substrates in organic solvents has been addressed by a number of researchers. For example, Halling, Jakubke, and coworkers found that chymotrypsin was capable of catalyzing efficient peptide bond formation in hexane even when the amino acid derivative substrates were used in their insoluble hydrochloride form.[5] Vulfson et al. utilized eutectic mixtures to aid in the interaction of insoluble substrates with insoluble enzymes.[6] In both these cases, the lack of a defined dissolved substrate solution clearly did not impede enzyme-catalyzed transformations, yet it remains unclear how direct substrate–enzyme interactions proceed. Recently, Paradkar and Dordick developed a method to solubilize enzymes in hydrophobic organic solvents through the formation of enzyme–surfactant ion pairs.[7] These ion-paired, organic-soluble enzymes are extremely active in hydrophobic solvents, such as isooctane.

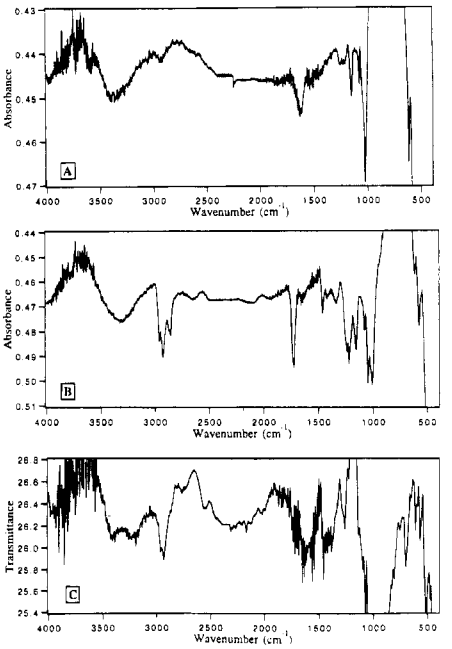

Figure 1. FT-IR spectra of (A) native amylose, (B) derivatized amylose on a ZnSe slide, and (C) derivatized cryogenically milled amylose powder.

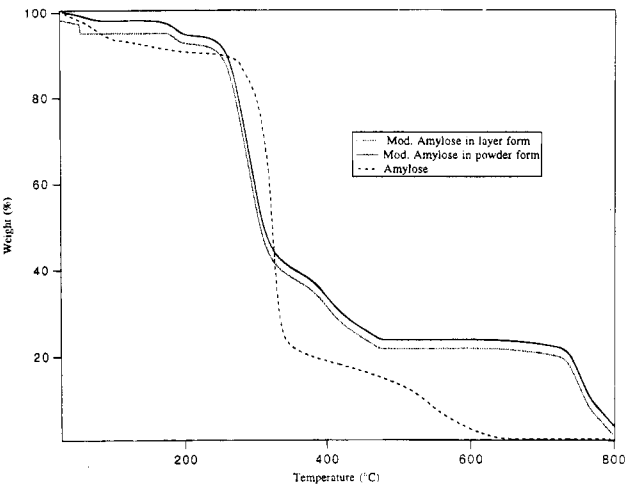

Figure 2. Thermogravimetric analysis of native and derivatized amylose.

In the present study, we show that amylose (an organic solvent-insoluble polysaccharide consisting of α-1,4-linked glucose moieties), when deposited as a thin film, can be regioselectively acylated by catalysis in organic solvents using an organic-soluble enzyme preparation of subtilisin Carlsberg (from *Bacillus licheniformis*). This represents the first attempt at catalyzing solvent-insoluble polymer modification using enzymes in organic solvents.

The enzymatic transesterification reaction was initiated by adding 60 mM vinyl caprate (C_{10}) to an isooctane solution containing solubilized subtilisin Carlsberg.[8] The solution, which contained a 40-fold molar excess of the vinyl ester relative to amylose hydroxyl groups, was pipetted onto a thin layer of amylose deposited onto ZnSe slides.[9] The reaction was allowed to proceed for 48 h at 37 °C, at which time the reaction was terminated

* To whom correspondence should be addressed.
† University of Iowa.
‡ U.S. Army Natick Research, Development, & Engineering Center.
§ University of Massachusetts at Lowell.
∥ Rohm & Haas Co.

0024-9297/95/2228-8881$09.00/0

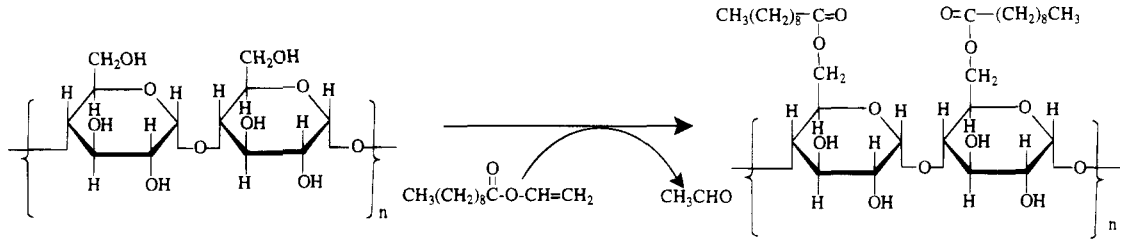

Figure 3. Proposed structure of modified amylose prepared via subtilisin Carlsberg catalysis.

by removing the solid amylose film and washing it with fresh isooctane to remove unreacted vinyl ester. FT-IR spectroscopy was used to determine whether enzymatic acylation of amylose occurred. As depicted in Figure 1, the enzymatic reaction resulted in a derivatized amylose with a spectrum that contained clear peaks at 2920 and 2850 cm^{-1}, corresponding to the methylene stretching mode of an alkyl chain in the amylose reaction product, as well as a peak in the region 1693−1760 cm^{-1}, corresponding to a C=O group. None of these peaks is present in the unmodified amylose or in amylose treated with the vinyl esters in the absence of enzyme or with enzyme predeactivated by boiling in water for 30 min prior to extraction and preparation in the soluble form (Figure 1). Moreover no vinyl group is present in the modified amylose, as detemined by the lack of absorbance at 900 and 1000 cm^{-1} in the FT-IR spectrum. Thus, nonselective adsorption of the vinyl esters to the amylose during the reaction does not occur.[10]

In addition to amylose film, cryogenically milled amylose powder was examined as an insoluble substrate in isooctane.[11] The FT-IR spectrum of the amylose powder following enzymatic reaction also showed clear evidence of acylation (Figure 1C). It is important to note that when subtilisin powder was used as a catalyst, no amylose acylation occurred. Specifically, in the presence of 60 mM vinyl caprate and 10 mg/mL of subtilisin Carlsberg powder, no evidence of amylose derivatization[12] was apparent when amylose was either used in the powder form or deposited onto ZnSe as a thin film. Thus, soluble enzyme is an absolute prerequisite for acylation of the insoluble polymer.

The degree of amylose substitution in the powdered form was determined by thermogravimetric analysis (TGA) and in the thin film by both TGA and ESCA (electron spectroscopy chemical analysis). Figure 2 depicts the TGA profile for native amylose and enzymatically acylated amylose film and powder. The only significant difference between the modified and native amylose preparations (prior to substantial thermal degradation) was the weight loss in the former at 180 °C, which is characteristic of alkyl chain degradation and is absent in the native amylose. Quantification of the weight loss of the modified amyloses as compared to the native amylose indicates that ca. 0.15 and 0.30 acyl chains are associated per glucose moiety of the amylose for the film and powder forms, respectively. Unfortunately, only surface accessible amylose chains can be enzymatically acylated. Such surface area may represent only a small fraction of the total amylose present in the reaction suspension. To characterize the inherent ability of subtilisin to catalyze the acylation of insoluble, yet surface accessible amylose, ESCA analysis of the top 100 Å of the amylose film was performed. This analysis indicated that the acylated surface had a degree of substitution of 0.9 ± 0.1 acyl chains per glucose moiety, based on the C:O ratio. It is tempting to speculate that this degree of substitution can be achieved if the enzyme is highly regioselective and can acylate only one hydroxyl per glucose unit in the amylose. Indeed, subtilisin is known to acylate primary hydroxyl groups on sugars[13] in organic solvents. Amylose contains free hydroxyls at the 2, 3, and 6 positions, the last being a primary hydroxyl. ^{1}H-NMR was used to determine the position of enzymic acylation of amylose. In comparison to underivatized amylose, the only significant shift occurred in the 6-hydroxyl proton.[10] Thus, subtilisin appears to be highly efficient in catalyzing the acylation of nearly all available primary hydroxyl groups on accessible amylose polymers. Figure 3 depicts the proposed structure of the modified amylose polymer using vinyl caprate as acyl donor.

The enzymatic process described herein can be envisioned as a new method for the synthesis and modification of polymers in nonaqueous media, even when the substrate is insoluble in the organic solvent. We expect this approach to be amenable to a wide range of enzymes and acyl donors. We are presently using this technique to derivatize other polysaccharides and hydroxylated polymers.

Acknowledgment. We thank D. Oblas for the ESCA characterization. F.F.B. and J.S.D. acknowledge financial support from the Army Research Office (DAAL-03-91-C-0034).

References and Notes

(1) For general reviews see: (a) Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194. (b) Klibanov, A. M. *Trends Biochem. Sci.* **1989**, *14*, 141. (c) Khmelnitsky, Yu. L.; Levashov, A. V.; Klyachko, N. L.; Martinek, K. *Enzyme Microb. Technol.* **1988**, *10*, 710. (d) Zaks, A.; Russell, A. J. *J. Biotechnol.* **1988**, *8*, 259.

(2) (a) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114 and references therein. (b) Faber, K. *Biotransformations in Organic Chemistry*; Springer-Verlag: Heidelberg, 1992 (see also references therein).

(3) Native enzymes are insoluble in nearly all organic solvents (Dordick, J. S. *Enzyme Microb. Technol.* **1989**, *11*, 194).

(4) Mayer, J. M.; Kaplan, D. L. *Trends Polym. Sci.* **1994**, 2, 227.

(5) Kuhl, P.; Halling, P. J.; Jakubke, H.-D. *Tetrahedron Lett.* **1990**, *31*, 5213.

(6) (a) Gill, I.; Vulfson, E. *Trends Biotechnol.* **1994**, *12*, 118. (b) Gill, I.; Vulfson, E. *J. Am. Chem. Soc.* **1993**, *115*, 3348.

(7) (a) Paradkar, V. M.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 5009. (b) Paradkar, V. M.; Dordick, J. S. *Biotechnol. Bioeng.* **1994**, *43*, 529. This approach is different from that used by several groups to dissolve proteins in organic solvents via covalent attachment of long, amphiphilic poly(ethylene glycol) tails onto the protein surface (Takahashi, K.; Tamaura, Y.; Kodera, Y.; Mihama, T.; Saito, Y.; Inada, Y. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 291).

(8) For this study, 1.1 mg/mL subtilisin Carlsberg was dissolved in HEPES buffer (8.5 mM, pH 7.8) containing 6 mM KCl. The aqueous solution was contacted with an equal volume of isooctane containing 2 mM Aerosol OT (AOT) and the two-phase solution was stirred at 250 rpm at 25 °C. After 30 min the phases were allowed to settle, the organic phase was removed, the water was removed by passing a stream

of N_2 through the solution,[7] and 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 dissolved in the isooctane solution which had a water content <0.01% (v/v).

(9) Amylose was dissolved in water and dried onto a ZnSe slide as a thin film. The reactions were performed with 1.0 mg/mL soluble subtilisin in isooctane containing 60 mM vinyl caprate in a beaker containing the ZnSe slide in the absence of shaking for 48 h at 37 °C. Amylose was pure and devoid of protein (as determined by FT-IR and the lack of detectable amide bonds). Amylose from the same batch was used in all experiments.

(10) ^{1}H-NMR of the enzymatically modified amylose powder shows 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 amylose. Such groups do not exist in the unmodified amylose. Enzymic specificity is demonstrated by ^{1}H-NMR (DMSO-d_6): *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, m, area 0.336), 5.5 (3 OH, br, area 0.330), 5.6 (2 OH, br, area 0.330) [similar to that in *The Polysaccharides* (Aspinall, G. O., Ed.; Academic Press: New York, 1982; Vol. 1, p 161]; *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). Note the shift of the 6-OH proton in the derivatized amylose. Furthermore, note that the area ratio of 6-OH proton to total protons in the native and derivatized amylose is 0.26 and 0.23, respectively. This provides additional evidence that acylation was confined at the 6-OH group. Integration of the alkyl chain protons and the amylose protons resulted in a calculated degree of substitution of 0.185 [following a method developed by: Itoh, T.; Tsujii, Y.; Suzuki, H.; Fukuda, T.; Miyamoto, T. *Polym. J.* **1992**, *24*, 641]. 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}H-NMR. It is important to note that chemically acylated amylose (using acyl chlorides) shows TGA and FT-IR results similar to those of enzymatically-treated amylose.

(11) The amylose powder had a particle size of less than 100 μm and was obtained by isolating milled amylose through a 100 μm screen. The surface area to weight ratio was 546 cm^2/g.

(12) No changes in the FT-IR and ^{1}H-NMR spectra were observed as compared to the native amylose.

(13) (a) Patil, D. R.; Rethwisch, D. G.; Dordick, J. S. *Biotechnol. Bioeng.* **1991**, *37*, 639. (b) Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 5849. (c) Rich, J. O.; Bedell, B. A.; Dordick, J. S. *Biotechnol. Bioeng.* **1995**, *45*, 426. (d) Plou, F. J.; Cruces, M. A.; Bernabe, M.; Martin-Lomas, M.; Parra, J. L.; Ballesteros, A. *Ann. N.Y. Acad. Sci.* **1995**, *750*, 332.

MA951240F