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Effects of Substitution Site on Acetyl Amylose Biodegradability by Amylase Enzymes

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ABSTRACT: The site-selective syntheses of water soluble (6-*O*)- and (2-*O*/3-*O*)-acetyl amylose polymers (substituted at primary and secondary hydroxyl functionalities, respectively) were carried out. On the basis of ^{1}H NMR analyses regiospecificities of >95% were achieved. In addition, routine chemical methods which did not employ protection−deprotection steps provided water soluble (2-*O*/3-*O*/6-*O*)-acetyl amylose polymers. To maintain water solubility, the polymer degree of substitution (ds) was maintained at <0.70. The biodegradation characteristics of these products as a function of site and ds were studied by exposures to the α-amylases from *Bacillus subtilis*, *Bacillus licheniformis*, and *Aspergillus oryzae*. Quantitation of the biodegradation rate and percent were carried out using the dinitrosalicylic acid (DNS) reducing sugar assay. Common to all three α-amylases was that these enzymes degraded (2-*O*/3-*O*)-acetyl amylose polymers much more rapidly and to greater extents than (6-*O*)-acetyl amylose derivatives of similar ds's and molecular weights (M_v). The rate of and percent degradation of (2-*O*/3-*O*/6-*O*)-acetyl amylose polymers was intermediate to that of (2-*O*/3-*O*)- and (6-*O*)-acetyl amylose polymers. Thus, the importance of site of substitution on the biodegradability of acetyl amylose polymers was demonstrated. Interestingly, when low ds (∼0.20) acetyl amylose polymers were exposed to the exoglycosidase from sweet potatoes (*β*-amylase), little to no polymer degradation was observed. This is believed to result from the rapid formation of substituted chain ends that are not degraded by the *β*-amylase, thus terminating further chain degradation events.

Introduction

Polysaccharides represent an exciting family of biodegradable polymers that hold great potential for providing a broad range of important functional properties. They are renewable resources synthesized by both plants and various microbial species and are the most abundant organic substances on earth.[1] However, their utility is limited because of (1) polymer thermal decomposition that precludes melting due to strong interchain hydrogen bonding interactions[2] and (2) high water sorptivity causing dramatic changes in material properties as a function of relative humidity.[3] Thus, a fundamental challenge presented by polysaccharides is how they can be modified so that biodegradability is not lost and desirable physical properties are achieved.

Polysaccharide esters offer the opportunity, in principal, to maintain material susceptibility to environmental degradability while providing a diverse range of physical properties. Recently, work in our laboratory and elsewhere has shown that cellulose esters are in fact biodegradable under a wide variety of conditions.[4−6] Specifically, it has been shown that cellulose acetates (CA) having a degree of substitution (ds) of less than ∼2.5 are biodegradable under both aerobic[4,5] and anaerobic[6] conditions. Also, many CA's were shown to be compostable.[5,7−10] The formation of side chain esters of cellulose has led to important thermoplastics.[11,12] Limitations encountered in using CA due to a relatively narrow $T_m - T_g$ gap (∼50 deg) have been overcome by the use of mixed side chain esters such as cellulose acetate propionate and cellulose acetate butyrate which have wider $T_m - T_g$ gaps and melting points.[13,14] Of

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importance in this respect is that workers at Eastman have recently shown by radiochemical labeling that cellulose propionates with a ds less than ~1.85 are biodegradable.[6] Our laboratory[15] and researchers at Eastman[6] have hypothesized that the biodegradation of CA of ds greater than 1 requires the work of a deacetylase as well as cellulase enzymes. It is likely that the deacetylase will degrade the polymer to a sufficiently low ds where the cellulase enzymes can begin degradation reactions between main chain sugar residues. Therefore, both deacetylase enzymes that cleave side chain polysaccharide esters as well as glycosidases that can degrade partially deacetylated polysaccharide products are of interest in trying to gain an understanding of the relationship between polysaccharide ester structure and enzyme biodegradability. Of relevance to the above discussion, this investigation considers the biodegradation of amylose acetate of low ds by α-amylase enzymes.

Amylose, the linear isotactic polymer component of starch,[16] represents an important polysaccharide to use as a model system to obtain information on the effect of polysaccharide modification on enzymatic degradation. Many types of enzymes have evolved which catalyze the hydrolysis of the (1→4) glycosidic linkages between residues in amylose. This broad range of enzymes, called amylases, is produced by animals, plants, and bacteria.[17] They consist of four broad categories, α-amylases, *β*-amylases, isoamylases, and amyloglucosidases. In addition, it is generally accepted that α-amylase enzymes may be classified into three different families according to their degradation mechanism, protein structure, and the structure of low molecular weight degradation products.[18,19] The Bacillus and Fungi families of α-amylase enzymes were included in this study. Specifically, α-amylases from *Bacillus subtilis*, *Bacillus licheniformis*, and the fungus *Aspergillus oryzae* were used. For the α-amylases produced by *A. oryzae* (Taka-amylase) and *B. subtilis*, both the primary amino acid sequence and the three-dimensional structure are known.[20−25]

Research on amylose esters has been carried out by several groups.[26−29] However, in all of these cases, the *O*-acylation was random and there has been limited study of the effect of *O*-acylation on biologically mediated degradation. In this paper, chemical methods were used to produce derivatives with control over the site and degree of *O*-acyl substitution. Specifically, (6-*O*)- and (2-*O*/3-*O*)-acetyl amylose polymers substituted predominantly at primary and secondary hydroxyl sites, respectively, were prepared. In addition, routine acetylation procedures were used to prepare acetyl amylose polymers substituted at all three sites (2-*O*/3-*O*/6-*O*). The structure and molecular weight of these amylose esters were studied by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, gel permeation chromatography (GPC), and dilute solution viscometry. The biodegradability of these derivatives differing in position and degree of acetylation was studied by exposure of these polymers to α-amylase enzymes from *B. subtilis*, *A. oryzae*, and *B. licheniformis*. In addition, studies were carried out using the *β*-amylase from sweet potatoes. The rate of and percent degradation (defined below) were measured by the dinitrosalicylic acid assay method.[30−34] Results of this work showed important differences in the degradability of acetyl amylose by α-amylases as a function of the site of amylose substitution.

Experimental Section

Materials. Pyridine was dried over calcium hydride (CaH_2) (Aldrich) for 24 h and subsequently distilled from CaH_2 under an argon atmosphere. The dried, distilled pyridine was stored under an argon atmosphere. Dimethyl sulfoxide (DMSO) was dried over CaH_2 for 24 h followed by vacuum distillation from CaH_2. Dried, distilled DMSO was stored over molecular sieves under an argon atmosphere. Acetic anhydride (reagent grade, 99+%), acetic acid (reagent grade, 99.8%), acetyl-d_3 chloride (reagent grade, 99+ atom % D), 3,5-dinitrosalicylic acid (98%), potassium sodium tartarate tetrahydrate (99.98%), phenol (99+%), and sodium metabisulfite (97%, ACS Reagent Grade) were all obtained from Aldrich and used as received.

Instrumental Methods. Nuclear Magnetic Resonance (NMR). Proton (^{1}H) NMR spectra were recorded on a Brüker WP-270 SY spectrometer at 270 MHz. ^{1}H NMR chemical shifts in parts per million (ppm) are reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The parameters for the polymer spectra are as follows: 4.0% (w/w) polymer in dimethyl sulfoxide-d_6 (DMSO-d_6), temperature 343 K, pulse width 4.9 s, 32K data points, relaxation delay 2.0 s, 150−200 transients. Peak areas were determined by spectrometer integration. They are reported as relative intensities representing a given number of hydrogens where the anomeric methine hydrogen (proton d, see Figure 1) is arbitrarily given a value of 1.00. The following abbreviations are used to present the ^{1}H NMR spectral results: s = singlet, db = doublet, tr = triplet, m = multiplet, br = broad.

Infrared Spectra (IR). Spectra were recorded on polymer/KBr pellets using a Brüker IFS 113v FT-IR at 25 °C. The following abbreviations are used to present the FT-IR spectral results: s = strong, vs = very strong, m = medium, w = weak, br = broad, sha = sharp, sh = shoulder.

Molecular Weight Measurements. Molecular weights were determined by GPC and dilute solution viscosity using a universal calibration curve. Studies by GPC were carried out using a Waters Model 510 pump, Model 410 refractive index detector, and Model 730 data module with KD-805, KD-803, and KD-801 Shodex columns in series. *N*,*N*-Dimethylformamide (DMF) containing 1% LiBr (w/v) was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 0.5% (w/v) and injection volumes of 100 μL were used. Dilute solution viscosity measurements on polymer samples and low polydispersity polystyrene standards (Aldrich) were carried out using an Ubbelohde viscometer in a constant temperature bath at 35 °C using DMF/LiBr as the solvent. A universal calibration curve was generated using GPC retention times and intrinsic viscosity measurements of low polydispersity polystyrene standards (Aldrich).

Elemental Analysis. Analyses were carried out by Atlantic Microlab, Inc., Newark, DE.

Enzyme Degradation Measurements. Enzyme−Substrate Incubations. The following enzymes were used to study the enzymatic degradation of the site-selective amylose ester derivatives: *B. subtilis* α-amylase from Sigma (Type II-A), *A. oryzae* α-amylase from Sigma (Type X-A), *B. licheniformis* α-amylase from Sigma (Type XII-A), and sweet potato *β*-amylase from Sigma (Type I-B). The units of activity of these enzymes were determined where 1 unit = 1 mg of reducing sugar liberated per mg of crude enzyme (in 3 min at 37 °C and pH 7) and were found to be 2.45, 2.37, 2.52, and 1.64, respectively. Enzyme concentrations used were 2.0, 2.5, 2.5, and 3.5 mg/mL, respectively. All degradation studies were carried out in triplicate at 37 °C in phosphate buffer at pH 7.0. Sodium azide was added (0.2 mg/mL) to the phosphate buffers to inhibit microbial growth. Substrates (15 mg/mL) either dissolved or suspended (20−40 mesh size) were incubated in the phosphate buffer−enzyme solutions for 0.5, 1, 2, 3, and 4 h. Each sample was then placed in a boiling water bath for 5 min to denature the α-amylase enzyme, cooled to room temperature, and then assayed as described below to determine the reducing sugar value.

Enzymatic Degradation Assay. Enzymatic degradation studies were carried out by measurement of reducing sugars

using the dinitrosalicylic acid (DNS) method.[30−34] DNS reagent was prepared by first dissolving 10.56 g of 3,5-dinitrosalicylic acid and 19.8 g of NaOH pellets in 1.416 L of distilled deionized water. A 306.0 g quantity of Rochelle salts (potassium sodium tartarate tetrahydrate), 7.62 mL of phenol, and 8.28 g of sodium metabisulfite were then added, and the reagent was aged for 2 weeks. Then 3 mL of DNS reagent was added to each enzyme−substrate solution subsequent to incubation and enzyme denaturation (see above), and the amount of reducing sugars present was obtained spectrophotometrically using a Bausch and Lomb spectrophotometer set at 550 nm. The calibration curve for determination of reducing sugars was generated by using high-purity maltose standards (Sigma).

Glucose Assay. The quantity of unsubstituted glucose residues was measured for acetyl amylose samples which had been converted selectively to the methyl ether derivative (see procedure below). The amylose methyl ether derivatives (10 mg/mL) were dissolved in 2.0 N sulfuric acid (100 mL) and degraded to monomeric units by heating at 100 °C for 6 h under an argon atmosphere. A D-glucose enzyme assay kit obtained from Boehringer Mannheim was used by following the manufacturers instructions[35−37] to obtain the mole fraction of unsubstituted glucose present after acidic hydrolytic degradation.

Synthetic Procedures. Starch Fractionation. Potato and high amylose (Hylon VII) starch types (supplied by Manischewitz Corp. and National Starch and Chemical Co., respectively) were fractionated following a literature method[38] to obtain amylose.

(6-*O*)-Acetyl Amylose. The synthesis of 6-*O*-acetyl amylose derivatives was carried out by following the procedure described by Horton and co-workers.[39] In summary, the method used involves the preparation of the per(trimethylsilyl) ether amylose derivative and subsequent reaction of this intermediate at 65 °C in carbon tetrachloride with acetic anhydride (2.5 mol of acetic anhydride/mol of persilylated glucose residues). Variable degrees of acetylation at the 6-*O* position were achieved by using different reaction times. Specifically, ds values of 0.24, 0.48, 0.65, and 0.91 were obtained by using reaction times of 1.5, 2.5, 4.0, and 5.5 days, respectively. Products were then disilylated by following the procedure described by Horton and co-workers[39] to form a series of 6-*O*-acetyl amylose derivatives. Characterization of the 6-*O*-acetyl amylose derivative with ds 0.65 is as follows: IR (KBr pellet) 3400 (vs, br), 2990 (s, br), 1745 (vs, sha), 1653 (s, sha), 1450 (s, sha), 1420 (s, br), 1375 (s, sha), 1153 (s, br), 930 (m, sha), 856 (m, sha), 764 cm^{-1} (m, sha); ^1H NMR (270 MHz, DMSO-d_6) [after reaction with acetyl-d_3 chloride (see below)] δ 2.09 (s, 1.95H), 5.23 (db, 1H). Calcd: C, 46.13; H, 6.04. Anal. Found: C, 46.07; H, 5.98.

(2-*O*/3-*O*)-Acetyl Amylose. The preparation of 2-*O*/3-*O*-acetylated amylose was carried out following a modification of the procedure described by Whistler and Hirase.[40] The method first required the preparation of the 6-*O*-trityl-protected amylose derivative which followed exactly as was previously described.[40] The trityl ds was determined to be 1.01 by ^1H NMR spectral integration of signals due to trityl phenyl hydrogens (7.0−7.3 ppm) and the anomeric methine hydrogens (5.2 ppm). Characterization of the 6-*O*-trityl amylose derivative with ds 1.01 is as follows: ^1H NMR (270 MHz, DMSO-d_6) δ 5.23 (br, 1H), 7.15 (m, 15.12H); IR (KBr pellet) 3050 (s, br), 2990 (s, br), 1995−1650 (w, br), 1490 (s, sha), 1450 (s, sha), 740 (m, br), 695 cm^{-1} (m, sha). Anal. Calcd: C, 74.23; H, 5.99. Found: C, 74.15; H, 5.92. Further confirmation of the degree of tritylation was obtained from ^1H NMR and elemental analysis of the butyl isocyanate derivative as follows: ^1H NMR (270 MHz, DMSO-d_6) δ 0.85 (tr, 2.87H), 5.23 (br, 1H), 7.15 (m, 15.19H). Anal. Calcd: C, 67.60; H, 6.97. Found: C, 67.55; H, 6.86.

To form secondary acetylated amylose derivatives, 6-*0*-trityl amylose (24.2 g) was first dissolved in dry pyridine (250 mL) and then acetic anhydride (28 mL) and acetic acid (15 mL) were added at 25 °C. The reaction was then carried out at 100 °C with vigorous mechanical stirring under an argon atmosphere. Aliquots (50 mL) were removed after 15, 30, 45, 60, 75, 90, 120, and 150 min to obtain products of variable degrees of acetylation. Each aliquot was added to 500 mL of methanol to precipitate the corresponding product, and the precipitates were washed four times with methanol (200 mL volumes) and dried (50 °C, 7 mmHg, 2 days). Detritylation was achieved by dissolution of products (2.0 g) in neat dichloroacetic acid (25 mL) and maintaining the solution at 25 °C for 25 min.[41] This mixture was then precipitated into cold methanol (500 mL) and the product collected by vacuum filtration. A sample dissolved in concentrated sulfuric acid did not yield tritylcarbinol as a precipitate upon dilution with water, indicating that detritylation was complete.[40] Characterization of the (2-*O*/3-*O*)-acetyl amylose derivative with ds 0.69 is as follows: IR (KBr pellet) 3400 (vs, br), 2990 (s, br), 1745 (vs, sha), 1653 (s, sha), 1450 (s, sha), 1420 (s, br), 1375 (s, sha), 1153 (s, br), 930 (m, sha), 856 (m, sha), 764 cm^{-1} (m, sha); ^1H NMR (270 MHz, DMSO-d_6) [after reaction with acetyl-d_3 chloride (see below)] δ 1.89 (s, 0.45H), 1.99 (s, 1.61H), 5.18 (db, 1H). Anal. Calcd: C, 47.43; H, 5.89. Found: C, 47.31; H, 5.76.

(2-*O*/3-*O*/6-*O*)-Acetyl Amylose. Amylose from Hylon VII starch (10 g) was dissolved in dry DMSO (500 mL) in a 1.0 L round bottom flask. A mixture of dry pyridine (100 mL), acetic anhydride (125 mL), and acetic acid (50 mL) were added, and the reaction mixture was stirred at ambient temperature under an argon atmosphere. Aliquots (175 mL) were withdrawn from the reaction vessel after 0.5, 1, 2, and 3 h and added to methanol (1 L). The resulting precipitates were isolated by vacuum filtration, washed with methanol and then a methanol:water 70:30 mixture, dried (60 °C, 7 mm Hg, 2 days), and stored in a desiccator over Drierite. ^1H NMR and IR spectra of the (2-*O*/3-*O*/6-*O*)-acetyl amylose products were similar to those described above for site-selective amylose derivatives of comparative ds.

***d_3*-Acetylation of Acetyl Amylose Unreacted Hydroxyl Functionalities.** (6-*O*)-, (2-*O*/3-*O*)-, and (2-*O*/3-*O*/6-*O*)-acetyl amylose products were further reacted with acetyl-d_3 chloride to obtain samples of almost complete (ds > 2.8) acetylation. As was previously reported,[42] synthesis of acetyl-d_3 derivatives is useful in simplifying acetyl amylose ^1H NMR spectra for determination of the ds. Derivatizations were carried out by addition of acetyl-d_3 chloride (0.5 mL) to acetyl amylose (100 mg) and subsequent reaction for 4 days at ambient temperature under argon with magnetic stirring. The reaction contents were then added to methanol to precipitate the products which were isolated by vacuum filtration, washed three times with methanol (100 mL portions) and dried (60 °C, 7 mmHg, 16 h). IR spectra of these products (KBr pellets) showed no evidence for the remaining unreacted hydroxyl side groups (complete absence of O−H stretching in the region 3400 cm^{-1}).

***O*-(1-methoxyethyl)-*O*-Methyl Amylose.** The site specific replacement of acetyl amylose acetyl groups with methyl ether substituents was carried out exactly as was previously described by deBelder and Norrman.[43] The products (0.25 g) were then subjected to acid hydrolysis in 2 N sulfuric acid (100 mL) for 6 h at 100 °C under an argon atmosphere to give a mixture of D-glucose and D-glucose methyl ethers which were analyzed by the D-glucose assay (see above).

Results and Discussion

Degrees of amylose substitution were maintained below 0.7 (water soluble derivatives) where the operative mechanism of environmental degradation will likely involve α-amylase-catalyzed chain cleavage events. Two basic synthetic strategies were followed to yield both primary (6-*O*) and secondary (2-*O*/3-*O*) site-selective acetylated amylose polymers. Specifically, the procedure described by Horton and co-workers was found to be an excellent approach which facilitated selective 6-*O* acetylation.[39] This method involved the heterogeneous persilylation of amylose followed by reaction with acetic anhydride under mild conditions. The resulting products were then desilylated to produce amylose derivatives selectively acetylated at the 6-*O* hydroxylic posi-

Scheme 1

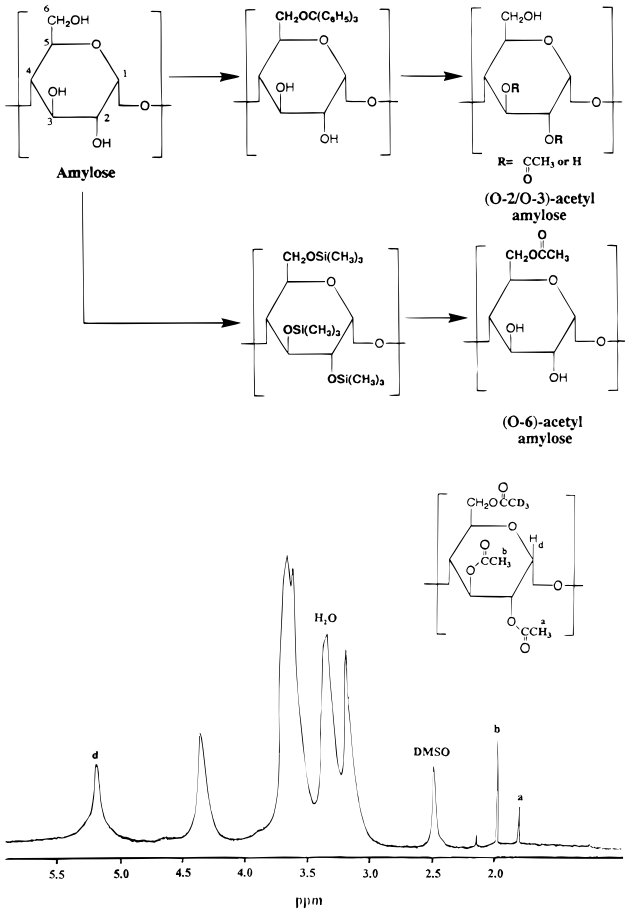

Figure 1. 270 MHz ^{1}H NMR spectrum in DMSO-d_6 at 70 °C of the acetyl-d_3 chloride derivatized (2-*O*/3-*O*)-acetyl amylose (ds 0.69) product.

tions (see below). O-acylation at the secondary hydroxylic positions was accomplished by following the procedure described by Whistler and Hirase.[40] This strategy involved the protection of the primary (6-*O*) hydroxylic groups by tritylation followed by *O*-acetylation and detritylation to produce amylose derivatives selectively acetylated at the 2-*O*/3-*O* hydroxylic positions (see below). In addition, (2-*O*/3-*O*/6-*O*)-acetyl amylose polymers were prepared.

Both the degree and site of acetylation for products was determined by recording ^{1}H NMR spectra of product acetyl-d_3 chloride derivatives (see Experimental Section). Derivatization of remaining unreacted hydroxyl groups with acetyl-d_3 chloride greatly facilitated ^{1}H NMR analyses by providing highly acetylated products which have approximately homogeneous repeat unit substitution. As an example, the ^{1}H NMR spectrum of acetyl-d_3 chloride derivatized (2-*O*/3-*O*)-acetyl amylose with a ds of 0.26 is shown in Figure 1. The ds values of acetyl amylose products were obtained by comparison of the spectral integration values for the protonated acetyl methyl groups (protons a, b, and c, 1.7−2.2 ppm, see Figures 1 and 2) and the anomeric methine hydrogen attached to C_1 (5.1−5.3 ppm). From this analysis, it was determined that (6-*O*)-, (2-*O*/3-*O*)-, and (2-*O*/3-*O*/6-*O*)-acetyl substituted amylose derivatives were prepared with ds values ranging from ∼0.2 to ∼1.0, where the maximum ds per glucose residue is 3.0 (see Table 1). Values of the ds determined by elemental analyses (see Table 1) were in excellent agreement with those obtained by ^{1}H NMR spectral integration. Specific ^{1}H NMR spectral assignments in this work for the acetyl

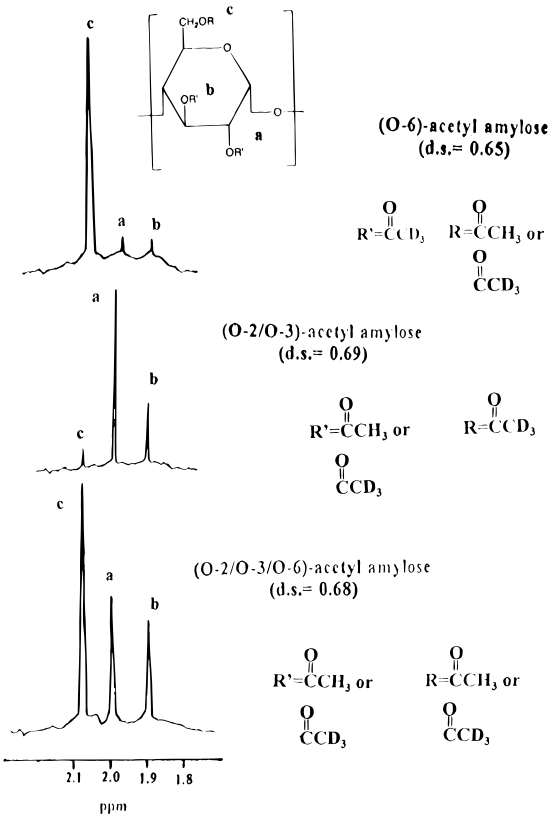

Figure 2. Expansions of acetyl methyl spectral regions for acetyl-d_3 chloride derivatized acetyl amylose polymers.

Table 1. Acetyl Amylose Polymer Molecular Weight and ds

acetyl amylose product[a]	ds[b]	ds[c]	MW[d]
(6-O)	0.19	0.18	24 000
(6-O)	0.24	0.22	23 100
(6-O)	0.37	0.35	23 500
(6-O)	0.48	0.49	22 500
(6-O)	0.65	0.69	23 900
(6-O)	0.69	0.66	22 500
(6-O)	0.91	0.87	21 000
(2-O/3-O)	0.23	0.23	25 000
(2-O/3-O)	0.25	0.24	24 500
(2-O/3-O)	0.41	0.40	22 500
(2-O/3-O)	0.47	0.49	23 500
(2-O/3-O)	0.61	0.64	22 200
(2-O/3-O)	0.65	0.66	21 000
(2-O/3-O)	0.99	1.02	19 400
(2-O/3-O/6-O)	0.35	0.33	14 500
(2-O/3-O/6-O)	0.68	0.64	8 500

[a] (X) where X designates sites substituted. [b] By ^{1}H NMR spectral integration (see Experimental Section). [c] From elemental analysis. [d] Molecular weight by GPC using a universal calibration curve (see Experimental Section).

group methyl resonances are based on that expected due to the protection−deprotection chemistry used herein and assignments made on related systems by Horton and Lauterbach[44] and Casu et al.[45] Expansions of the spectral region corresponding to the protonated acetyl methyl groups for (6-*O*)-acetyl (ds 0.65), (2-*O*/3-*O*)-acetyl (ds 0.69), and (2-*O*/3-*O*/6-*O*)-acetyl (ds 0.68) amylose polymers are shown in Figure 2. The acetyl methyl spectral region of the 6-*O*-substituted amylose derivative has one predominant signal at 2.09 ppm assigned to protons c. The 2-*O*/3-*O*-acetyl derivatives show two predominant acetyl methyl peaks at 1.89 and 1.99 ppm assigned to protons b and a, respectively. The spectrum of 2-*O*/3-*O*/6-*O*-acetylated samples showed substantial substitution at all three sites (see Figure 2). Thus, based on the ^{1}H NMR analyses presented, it appears

Table 2. Rate and Percent Degradation of Acetyl Amylose Polymers by Amylase Enzymes

acetyl amylose product[a]	amylase source	rate[b] (% degrad/h)	% degrad[c]	correlation[d] coefficients (r^2)
amylose[e]	*A. oryzae*	10	95(±15.2)	0.97
(6-O)-0.87	*A. oryzae*	0.33	6.1(±3.1)	0.94
(6-O)-0.69	*A. oryzae*	0.94	14.9(±3.7)	0.92
(6-O)-0.37	*A. oryzae*	1.6	22.4(±10.1)	0.97
(6-O)-0.19	*A. oryzae*	1.7	21.2(±8.6)	0.97
(2-O/3-O)-0.91	*A. oryzae*	0.61	8.1(±3.5)	0.93
(2-O/3-O)-0.65	*A. oryzae*	2.4	42.8(±4.8)	0.91
(2-O/3-O)-0.41	*A. oryzae*	5.0	73.2(±11.4)	0.94
(2-O/3-O)-0.25	*A. oryzae*	5.3	76.59(±12)	0.98
(2-O/3-O/6-O)-0.68	*A. oryzae*	1.47	15.9(±4.1)	0.91
(2-O/3-O/6-O)-0.35	*A. oryzae*	3.45	28.9(±4.6)	0.93
amylose[e]	*B. subtilis*	11	94(±13.3)	0.95
(6-O)-0.91	*B. subtilis*	0.52	3.1(±2.3)	0.95
(6-O)-0.65	*B. subtilis*	1.1	15.05(±8.2)	0.98
(6-O)-0.48	*B. subtilis*	1.4	17.15(±7.6)	0.98
(6-O)-0.24	*B. subtilis*	1.3	18.8(±9.5)	0.92
(2-O/3-O)-0.99	*B. subtilis*	0.96	7.5(±3.1)	0.97
(2-O/3-O)-0.61	*B. subtilis*	3.1	36.5(±8.9)	0.99
(2-O/3-O)-0.47	*B. subtilis*	5.2	70.7(±10.1)	0.99
(2-O/3-O)-0.23	*B. subtilis*	5.4	72.6(±10.9)	0.96
(2-O/3-O/6-O)-0.68	*B. subtilis*	1.31	15.2(±4.3)	0.93
(2-O/3-O/6-O)-0.35	*B. subtilis*	3.17	27.6(±4.5)	0.92
amylose[e]	*B. subtilis*	8.3	95(±12.0)	0.91
(6-O)-0.87	*B. licheniformis*	0.15	4.2(±2.9)	0.95
(6-O)-0.69	*B. licheniformis*	0.28	9.85(±7.6)	0.98
(6-O)-0.37	*B. licheniformis*	0.37	10.0(±7.7)	0.99
(6-O)-0.19	*B. licheniformis*	0.39	10.2(±12.0)	0.92
(2-O/3-O)-0.91	*B. licheniformis*	0.56	7.5(±3.5)	0.97
(2-O/3-O)-0.65	*B. licheniformis*	0.72	19.25(±7.0)	0.99
(2-O/3-O)-0.41	*B. licheniformis*	1.4	35.9(±8.9)	0.98
(2-O/3-O)-0.25	*B. licheniformis*	1.4	35.1(±8.2)	0.94
amylose[e]	sweet potatoes[f]	4.9	90(±12.0)	0.96
(6-O)-0.19	sweet potatoes	0.15	3.91(±3.4)	0.99
(2-O/3-O)-0.25	sweet potatoes	0.17	4.23(±2.9)	0.98

[a] (X)-*Y* where X designates sites substituted and *Y* the ds determined by ^{1}H NMR spectral integration. [b] Calculated from the initial slope of the % degradation vs time curve. [c] % degrad = (final experimental reducing sugar value)/(theoretical reducing sugar value for complete degradation to maltose or substituted maltose products) × 100. Standard deviation from the mean values reported are calculated from five repetitions for each sample. [d] Obtained from a linear regression analysis of the initial slope for the corresponding percent degradation vs time curve. [e] Not acetylated. [f] β-amylase.

that high regiospecificity (>95%) was achieved during the preparation of the 6-*O* and 2-*O*/3-*O* acetylated amylose derivatives.

The molecular weights of the acetylated amylose derivatives were analyzed by GPC using a universal calibration curve (see Experimental Section). This provided absolute viscosity average molecular weight (M_v) values. The M_v values of the site-selectively modified acetyl amylose derivatives were all similar, ranging from ∼19 000 to ∼25 000 (see Table 1). Thus, the introduction of polymer molecular weight as an additional variable for comparative degradation rates of site-selectively modified acetyl amylose polymers was minimized. The (2-*O*/3-*O*/6-*O*)-acetyl amylose derivatives had slightly lower M_v values. Therefore, the discussion below on the enzymatic degradation rates for these slightly lower molecular weight derivatives considers molecular weight variations.

Incubation of the synthesized acetyl amylose derivatives with α-amylase enzymes from different sources was carried out as was described above in the Experimental Section, and the results are compiled in Table 2. All acetyl amylose derivatives prepared with the exception of those with ds values of ∼0.9 and greater were soluble in the enzyme−substrate aqueous incubation solutions. The rate and percent of acetyl amylose degradation was monitored by assaying the reducing sugar value (in milligrams) as a function of time (see Experimental Section). The rate values were taken as the initial slope determined by linear regression of the line generated by plots of percent degradation verse time (see Figures 3−7). Percent degradation values were determined as follows:

$$\% \text{ degradation} = \frac{\text{final experimental reducing sugar value}}{\text{theoretical reducing sugar value}} \times 100$$

where the theoretical reducing sugar value is that calculated for complete degradation of the acetyl amylose polymers to maltose or substituted maltose products. Buffer solutions without addition of enzyme and using amylose as the substrate served as a control. No degradation was observed in the control sample so that degradation values attained in the presence of enzyme are attributed to enzyme catalyzed processes.

Results from incubations of site-selective acetyl amylose derivatives of relatively lower and higher ds with the α-amylase from *A. oryzae* are shown in Figures 3 and 4, respectively.

As was expected, amylose which was not substituted showed the fastest rate (percent degradation/h) and percent degradation. Also, the measured reducing sugar value for amylose after a plateau was reached corresponds closely to that calculated for the complete degradation of amylose chains to maltose (theoretical value = 15.8 mg). Most importantly, dramatic differences in the rate and percent of degradation were observed as a function of the site of substitution. Specifically, the secondary (2-*O*/3-*O*)-acetylated derivatives showed a faster rate and larger percent degradation than the primary (6-*O*)-acetylated derivatives for

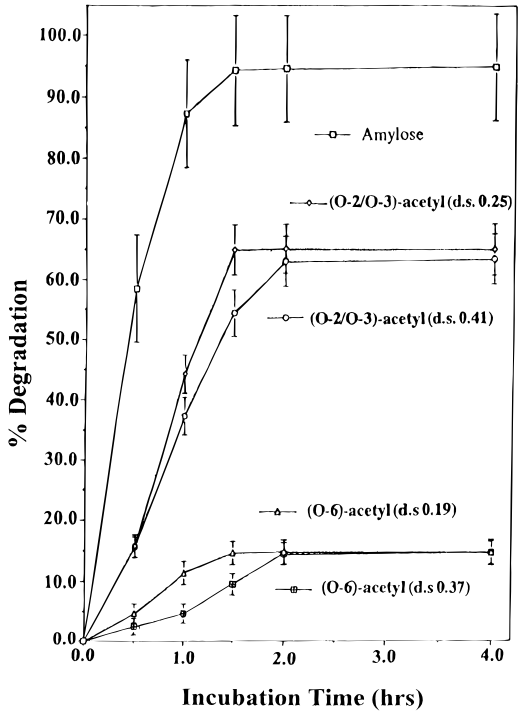

Figure 3. Percent degradation as a function of time for unsubstituted amylose and (6-*O*)- and (2-*O*/3-*O*)-acetyl amylose polymers exposed to the α-amylase from *A. oryzae*.

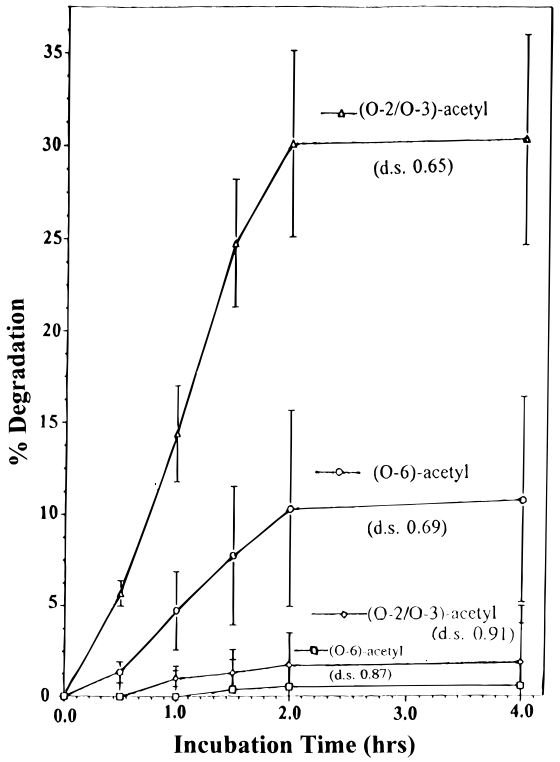

Figure 4. Percent degradation as a function of time for (6-*O*)- and (2-*O*/3-*O*)-acetyl amylose polymers exposed to the α-amylase from *A. oryzae*.

samples of almost identical ds and molecular weight (M_v). The percent degradation is between 3- and 5-fold higher for the 2-*O*/3-*O* derivatives compared to the 6-*O* derivatives. Similarly, enhanced degradation rates for the 2-*O*/3-*O* derivatives were also measured when the α-amylases from *B. subtilis* and *B. licheniformis* were used (see Table 2). Therefore, the higher activity of α-amylase enzymes for amylose which is acetylated at the 2-*O*/3-*O* positions appears thus far to be common to at least three different α-amylase enzymes from two different families. The low rate and percent of biodegradation for (6-*O*)- and (2-*O*/3-*O*)-acetyl amylose of ds ~ 0.9 (see Figure 4 and Table 2) may, in part, be due to the insolubility of these substrate polymers in the enzyme−substrate incubation solutions. Alternatively, α-amylase enzymes may be incapable of degrading modified acetyl amylose substrates with ds values approaching 1.0.

It is also interesting to consider whether there were substantial differences in the percent degradation observed as a function of the enzyme used for acetyl amylose substrates that had almost identical ds's and substitution sites. This analysis was simplified by the fact that the activities and concentrations of the α-amylase preparations used herein were of similar values (see the Experimental Section). On the basis of comparison of percent degradation values in Table 2 it was concluded that the relative extents of degradation of acetyl amylose substrates occurred as a function of the amylase used in the following order (from highest to lowest): *A. oryzae* ≅ *B. subtilis* > *B. licheniformis*.

The degradability of the primary and secondary acetyl derivatives when incubated with the exoglycosidase from sweet potatoes (*β*-amylase) was also studied. Interestingly, even for derivatives of low substitution (~0.20), little to no degradation of the substrate polymers was measured (see Table 2). One possible explanation for this result is that chain cleavage of modified amylose by exoglycosidases occurring at the chain end will lead to the formation of substituted chain end repeat units for low extents of degradation. These substituted chain ends are apparently not degraded by the exoglycosidase, thus terminating further chain degradation events.

Upon further inspection of the above results a question arises as to the relative mole fraction of free sugar repeat units for primary versus secondary substituted amylose of similar ds's. For the 6-*O*-acetyl derivatives, to a reasonable approximation, only one (the 6-*O*) hydroxylic functionality can react in each glucopyranosyl residue. However, for the 2-*O*/3-*O*-acetyl derivatives, there are two secondary hydroxylic functionalities which may react in each residue. This means that at similar ds values for 6-*O*- and 2-*O*/3-*O*-acetyl amylose polymers, there are likely a relatively greater number of unsubstituted glucose residues for the latter modified acetyl amylose polymers. For example, at a ds of 1.0 for selective 6-*O*-acetylation, all of the 6-*O* hydroxylic functionalities are derivatized so that no unsubstituted D-glucose residues remain. In contrast, an 2-*O*/3-*O*-acetyl derivative with a ds of 1.0 may, in the most extreme case, have up to 50% unsubstituted D-glucose residues. Of course, a preference for the formation of disubstituted relative to monosubstituted sugar residues for 2-*O*/3-*O* substitution is unlikely. However, a higher mole fraction of unsubstituted rings for 2-*O*/3-*O*- when compared to 6-*O*-acetylated amylose might provide an explanation for the relatively faster observed rates and percent of enzymatic degradation of the former substituted polymers. For example, it has been shown for the cellulase-mediated degradation of cellulose derivatives that a single substituent on each glucose unit affords complete resistance to enzymatic degradation.[46] Indeed, studies on carboxymethyl cellulose indicate that enzymatic cleavage occurs between two adjacent unsubstituted residues.[47,48] To address this question, the mole fraction of unsubstituted glucose residues for selected

Table 3. Comparison Between ds and the Mole Fraction of Substituted Rings for (2-O/3-O)-Acetyl Amylose Derivatives

ds[a]	sub. rings (mol fraction)[b]	ds[a]	sub. rings (mol fraction)[b]
0.23	0.20	0.61	0.51
0.47	0.41	0.99	0.86

[a] From ^{1}H NMR spectral integration. [b] Calculated by enzymatic assay of unsubstituted glucose after acid hydrolysis of the *O*-methyl amylose derivative (see Experimental Section).

6-*O*- and 2-*O*/3-*O*-acetyl amylose derivatives was determined. This was carried out by first selectively exchanging the acetyl side groups for methyl ether groups[43] followed by acid hydrolysis of the polymer (see Experimental Section). After degradation, the samples were assayed for the amount of unsubstituted glucose residues (see Experimental Section). The results, shown in Table 3, indicate that up to the ds value of ~0.47 there is very little difference in the mole fraction of substituted rings calculated by assuming that disubstituted 2-*O*/3-*O* residues do not exist (value equal to the ds) and the experimental value determined by the glucose assay. At ds values of 0.61 and higher, the difference in the mole fraction of substituted glucose residues based on ds and the unsubstituted glucose assay becomes significant (see Table 3). However, it is clear by review of the results above that the difference in unsubstituted sugar rings between 2-*O*/3-*O*- and 6-*O*-substituted polymers of similar ds's cannot explain the dramatically different degradation behavior of these respective derivatives. For example, the 2-*O*/3-*O*- and 6-*O*-acetyl amylose derivatives of ds 0.41 and 0.25, respectively, have values for the mole fraction of substituted rings of 0.36 and 0.22, respectively. Observation of Figure 3 (degradation by *A. oryzae* α-amylase) shows that the 2-*O*/3-*O* sample with ds 0.41 degrades at a faster rate and greater percent (~5 times) than the 6-*O* ds 0.19 sample even though the latter has a much greater number of unsubstituted glucose residues. Furthermore, comparison of the relative degradation rates by *A. oryzae* α-amylase of the 2-*O*/3-*O* ds 0.25 and the 6-*O* ds. O.19 samples shows dramatic differences in the rate of and percent degradation for only small differences in the mole fraction of unsubstituted glucose rings (see Tables 2 and 3). The same conclusion is reached by applying similar logic to the differences in the rate of and percent degradation resulting from exposures of 2-*O*/3-*O* and 6-*O*-acetyl amylose substituted samples to the α-amylase from *B. subtilis* (see Table 2). Also, on the basis of the above discussion, we believe that the results shown in Figure 4 and Table 2 for differences in degradability for 2-*O*/3-*O*- and 6-*O*-acetyl amylose substituted samples with ds values between 0.61 and 0.69 are due to the site of substitution and not differences in the mole fraction of unsubstituted sugar residues. Another consideration that further demonstrates that the mole fraction of unsubstituted sugar residues has little to do with the observed changes in degradation as a function of substituent position is the fact that large increases in ds for 2-*O*/3-*O* and 6-*O* acetyl amylose derivatives caused only small changes in rate and no significant change in percent degradation for both *A. oryzae* and *B. subtilis* α-amylases (see Figure 3 and Table 2). For example, upon a decrease in the ds of 6-*O*-acetyl amylose derivatives from 0.37 to 0.19 there was no appreciable change in the corresponding percent degradation by the α-amylase from *A. oryzae* (see Figure 3) while the mole fraction of unsubstituted rings increased from 0.63 to 0.81.

It has been shown by others that reactivity at the hydroxyl groups of polysaccharides such as starch and cellulose follows the order 6-*O* > 2-*O* > 3-*O*.[49] In our work for the preparation of (2-*O*/3-*O*)-acetyl amylose from 6-*O*-trityl amylose, reactivity of the 2-*O* hydroxyl was much greater than at the 3-*O* position such that for a ds of 0.69, the ratio of 2-*O*- to 3-*O*-acetylation was approximately 4:1 (see Figure 2). This high selectivity for the 2-*O* position may be attributed to the inherently higher reactivity of the 2-*O* position in combination with steric interactions between the bulky triphenylmethyl group at 6-*O* and the neighboring 3-*O* site. The relatively low reactivity of the 3-*O* position of trityl amylose explains the low percent of disubstituted sugar residues for the site-selective (2-*O*/3-*O*)-acetyl amylose derivatives (see Table 3). Of greater importance is that the high selectivity achieved at the 2-*O* position suggests that (2-*O*)-acetyl amylose is more susceptible to α-amylase degradation than (6-*O*)-acetyl amylose. It would be interesting in future work to synthesize and study the relative degradability of a predominantly (3-*O*)-acetyl amylose.

Our attention was then directed toward the degradability of (2-*O*/3-*O*/6-*O*)-acetyl amylose. Inspection of Figure 2 shows that acetylation to obtain (2-*O*/3-*O*/6-*O*)-acetylated products resulted in high selectivity at the 6-*O* position. Using as an example the (2-*O*/3-*O*/6-*O*)-acetyl product with a ds of 0.68, the d.s values at the 6-*O*, 2-*O*, and 3-*O* positions are 0.41, 0.16, and 0.11, respectively. Thus, (2-*O*/3-*O*/6-*O*)-acetyl amylose derivatives synthesized herein more closely approximate (6-*O*)- than (2-*O*/3-*O*)-acetyl amylose. Therefore, one might predict that (2-*O*/3-*O*/6-*O*)-acetyl amylose would degrade at a rate which is greater than that for (6-*O*)-acetyl amylose but much slower than that for (2-*O*/3-*O*)-acetyl amylose. In other words, based on the structure of (2-*O*/3-*O*/6-*O*)-acetyl amylose, it would be expected that its susceptibility to α-amylase degradation would more closely resemble that of (6-*O*)-acetyl amylose than of (2-*O*/3-*O*)-acetyl amylose. The relative degradability of these derivatives upon exposure to the α-amylase from *A. oryzae* is shown in Figures 5 and 6 (see also Table 2).

The results obtained for the relative degradability of (2-*O*/3-*O*/6-*O*)-acetyl amylose are precisely as was anticipated on the basis of the selectivity of substitution. The same conclusions were reached on the basis of the degradation results reported in Table 2 for exposures of these identical samples to the α-amylase from *B. subtilis*.

Since the relatively lower molecular weights of (2-*O*/3-*O*/6-*O*)-acetyl amylose derivatives introduces polymer molecular weight as an additional variable (see Table 1), studies were carried out to determine if there is an apparent effect of molecular weight (within the molecular weight range of interest to this study) on the observed enzyme degradation of acetyl amylose. Specifically, relatively high (20 000) and low (5000) molecular weight (2-*O*/3-*O*)-acetyl amylose derivatives were exposed to the α-amylase from *A. oryzae*. The results shown in Figure 7 at two extents of substitution (ds ~0.46 and ~0.63) indicate that changes in molecular weight between 5000 and 20 000 had no statistically significant effect on the rate of or percent degradation. Similar results were obtained when these identical samples were exposed to the α-amylase from *B. subtilis* (results not shown). Therefore, it appears that the

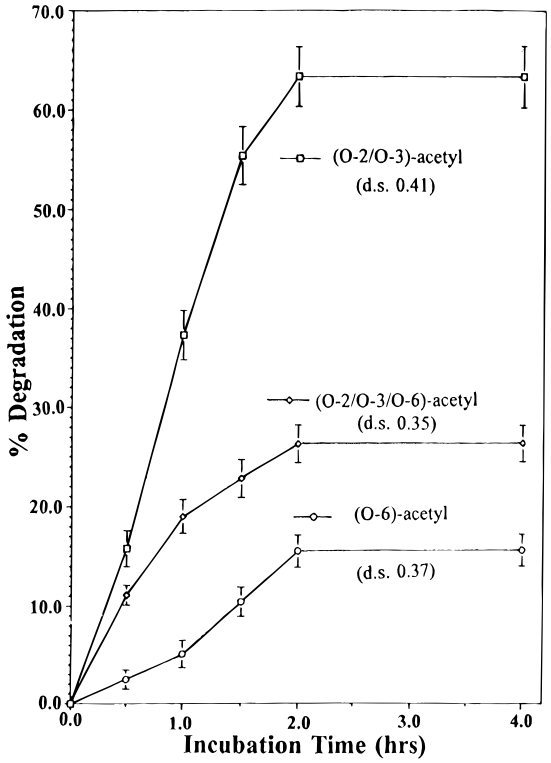

Figure 5. Percent degradation as a function of time for (6-*O*)-, (2-*O*/3-*O*)-, and (2-*O*/3-*O*/6-*O*)-acetyl amylose polymers exposed to the α-amylase from *A. oryzae*.

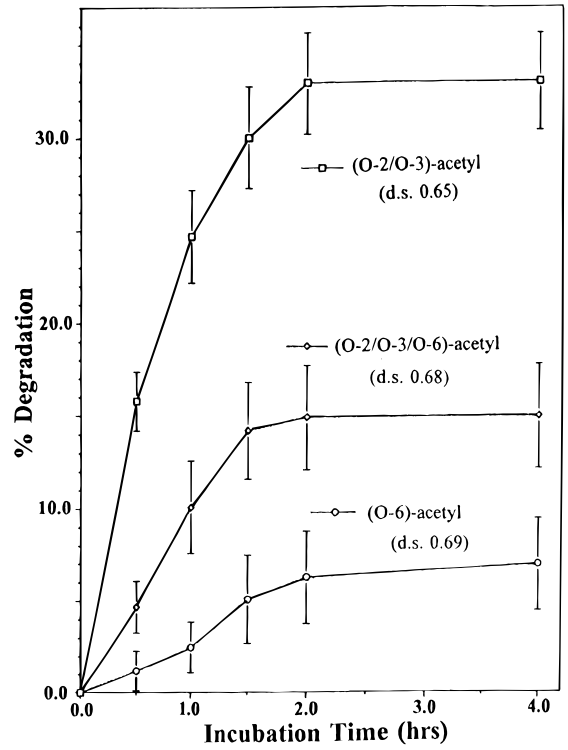

Figure 6. Percent degradation as a function of time for (6-*O*)-, (2-*O*/3-*O*)-, and (2-*O*/3-*O*/6-*O*)-acetyl amylose polymers exposed to the α-amylase from *A. oryzae*.

predominant 6-*O*-acetyl substitution for (2-*O*/3-*O*/6-*O*)-acetyl amylose polymers synthesized by routine methods results in decreased susceptibility to α-amylase degradation relative to (2-*O*/3-*O*)-substituted polymers.

It should be noted that (2-*O*/3-*O*/6-*O*)-acetyl amylose relative to (6-*O*)- and (2-*O*/3-*O*)-acetyl amylose is expected to have the highest value of unsubstituted

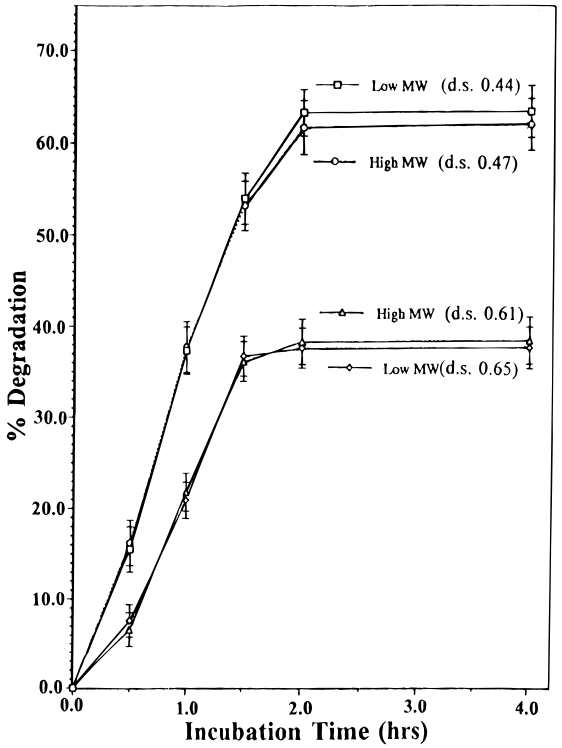

Figure 7. Percent degradation as a function of time for (2-*O*/3-*O*)-acetyl amylose polymers of 20 000 (denoted as high MW) and 5000 (denoted as low MW) exposed to the α-amylase from *A. oryzae*.

glucose rings for a given ds. Thus, the magnitude of decrease in degradability demonstrated above for (2-*O*/3-*O*/6-*O*)- relative to (2-*O*/3-*O*)-acetyl amylose would be smaller if effects of unsubstituted sugars were deemed significant.

Summary and Mechanistic Considerations

This work demonstrated that for the α-amylases studied herein, biodegradation of water soluble acetyl amylose proceeds at faster rates and to greater percent when acetylation of the polymer is at secondary 2-*O*/3-*O* as opposed to primary 6-*O* hydroxyl functionalities. Interestingly, these results were obtained using α-amylase enzymes from two distinct families (Bacillus and Fungi). The observed differences in rate as a function of substitution site must be due to changes in enzyme−substrate binding (K_m) constants and/or maximum velocity (v_{max}) values. Future work will report on K_m and v_{max} values for specific α-amylase−acetyl amylose pairs and will test existing information on the structure of α-amylase catalytic sites based on enzyme−substrate molecular modeling and the experimental data obtained herein. For the purposes of the present discussion it is interesting to consider that the three-dimensional enzyme−substrate binding site of the α-amylase from *A. oryzae* (Taka-amylase) has been investigated by X-ray structure analysis at 3 Å resolution by Matsuura et al.[20−22] Their studies showed that the histidine residues at positions 122 and 210 may form important hydrogen bonds with the 6-*O* hydroxyl groups of the substrate glucose residue at the binding site. Indeed, they believe that the hydrogen bond between the histidine residue at position 122 may be essential for the catalytic cleavage reaction to take place.[50,51] Our results appear supportive of this hypothesis. However, it should be noted that the work of Matsuura et al.[20−22] also shows hydrogen-bonding interactions between 2-*O*/3-*O* hy-

droxyl substrate functionalities and the α-amylase catalytic site so that at this stage of our work it is not possible to present conclusive statements as to the relationship between substituted amylose biodegradation rates and enzyme structure.

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