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Effect of Degree of Substitution of Octenyl Succinate Starch on Enzymatic Degradation

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Octenyl succinate starch of degree of substitution (ds) 0.03, 0.07, and 0.11 was synthesized in an aqueous medium. These compounds were then tested for the susceptibility to enzymatic degradation. The multiple-enzyme regime of α-amylase, amyloglucosidase, and pullulanase was chosen for the evaluation. This combination of enzymes had been proven to degrade 99.5% of unmodified starch to glucose and hence was chosen for this study. It was found that even small amounts of subsituent caused a considerable decrease in the extent of degradation. The net extent of degradation decreased with increasing ds. Surprisingly, the amount of glucose from all three substituted substrates was quite similar, suggesting the effect small amounts of subtituent had on the enzymatic activity.

KEY WORDS: Octenyl succinate starch; degree of substitution; enzymatic degradation.

INTRODUCTION

Starch is composed of two distinct fractions, amylose and amylopectin. Amylose consists of predominantly linear glucose units connected by predominantly α-1,4-glucopyranoside linkages, with a minor fraction of 1,6-linkages [1]. Amylopectin has the same glucose main-chain repeat units, but in addition, there are 1,6-linked side-chain branches for every 15 to 45 main-chain units. Depending on the botanical source of starch, different ratios of amylose to amylopectin can be obtained, with the most common amounts being about 60–80% amylopectin and the rest amylose. These glucan polymers show a molecular weight of from 250,000 to 1.5 million g/mol for the linear fraction [2].

Modifications of starch to tailor its properties for various applications has been of interest for many years [3]. These modifications disrupt hydrogen bonding and reduce retrogradation while also imparting other properties such as the introduction of hydrophobicity, melting to facilitate processing, metal chelating groups, etc. Such modified starches find uses in the paper industry as sizing agents, coating agents, adhesives, films, food additives, etc. The method of modification depends on the final application. For example, for the paper industry, modification in organic media is suitable. In contrast, for foods, modifications in organic media are not acceptable. Nowadays, there is a growing need to avoid solvents completely or to use environmentally friendly solvents such as water and liquid CO_2.

The most common enzymes available for the digestion of starch are α-amylases. α-Amylases are available from a wide variety of sources such as fungi, bacteria, mammals (e.g., pig and human), etc., and they differ in their physical structure and action pattern [4]. These enzymes share the characteristic that they can hydrolyze α-1,4-glucosidic linkages exclusively. This led to the postulate that a common catalytic mechanism must exist for all α-amylases [5].

Starch consists of α-1,4-linkages interspersed with α-1,6-linkages which provide a tree-like structure (Fig. 1). α-Amylases are capable of hydrolyzing the 1,4-linkages but are unable to cleave the 1,6-linkages [4]. This usually gives rise to several limit dextrins as the hydrolysis products.

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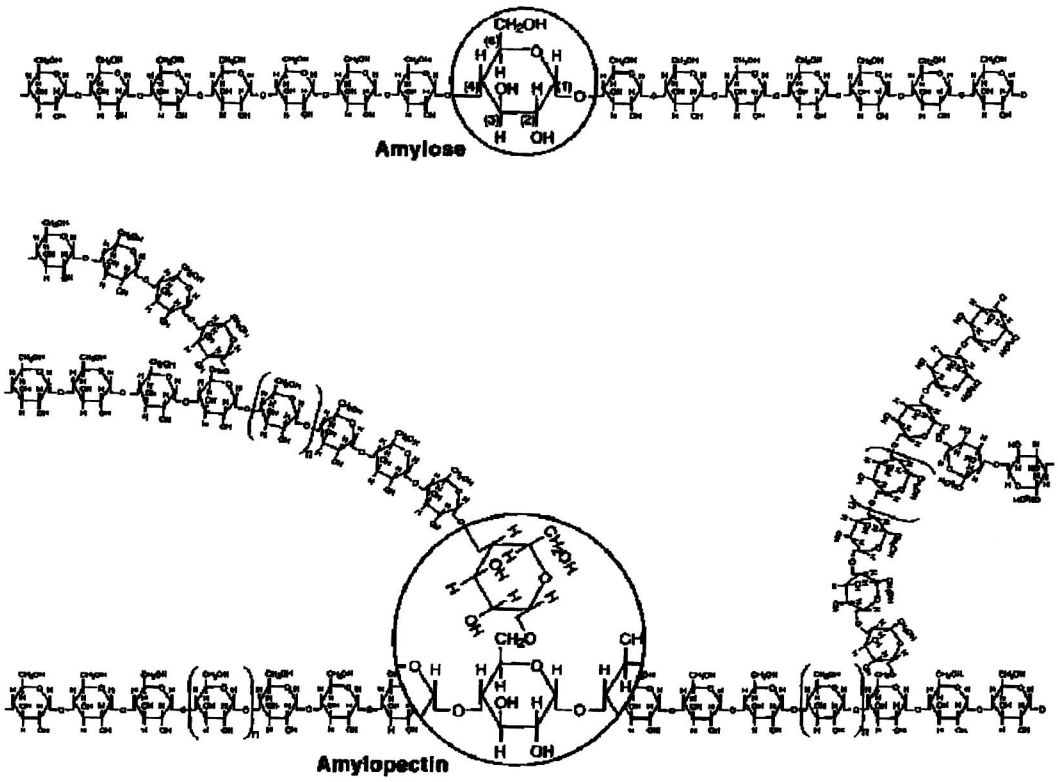

Fig. 1. Arrangement of the starch chains in the starch granule.

Amino acid composition and sequence information for most α-amylases is available, α-Amylases are known to contain several amino acid residues, the sequencing of which is unique for α-amylase from different sources. However, a high degree of homology has been observed, of which the residues 95–101, 193–201, and 295–301 are conserved in most α-amylases [5]. The α-amylases contain a cleft in the left-hand side of the molecule which is held together by a calcium ion chelated to several groups. The chains enter through the bottom of the cleft during hydrolysis.

Based on their studies, Matsuura *et al.* [6] have proposed an intermediate between the enzyme and the starch substrate involving 7 glucose units at any given time (Fig. 2). Among these amino acid residues, Glu230 plays the most important role in that it cleaves the acetal bonds between the 4 and the 5 glucose units. Figure 3 shows the sites of the enzyme that participate in the hydrolysis process. It has also been noted that His296, Asp297, His122, Glu230, Asp206, Val231, His210, Lys209, and Tyr155 are conserved in the α-amylases from porcine pancreas and *Aspergillus oryzae*.

Tao *et al.* [5] have observed a covalent linkage between the carboxylate group on the enzyme and the carbon 1 of the glucose unit. This linkage is then cleaved in the presence of water to regenerate the active carboxyl group of the enzyme and to give a reducing alcohol on the glucose ring (Fig. 3). This observation was made using low-temperature NMR studies with ^{13}C-labeled maltotetraose as the substrate. The carboxylate groups must presumably be from the Glu230 in the enzyme, while Asp297 is thought to be the electrophile.

Enzymes are also classified as being either exo- or endo-enzymes. Exo-enzymes are those that start cleaving the chain from one end, while endo-enzymes catalyze cleavage at random sites along the chain. α-Amylases are endo-enzymes, while β-amylases are exo-enzymes. Again, both of these enzymes are capable of hydrolyzing the α-1,4-linkages alone, which results in a number of α-limit dextrins. These enzymes are widely used to solubilize starch.

Pullulanases are a class of enzymes that are capable of hydrolyzing exclusively 1,6-linkages. These are endo-type enzymes that hydrolyze any of the 1,6-linkages in

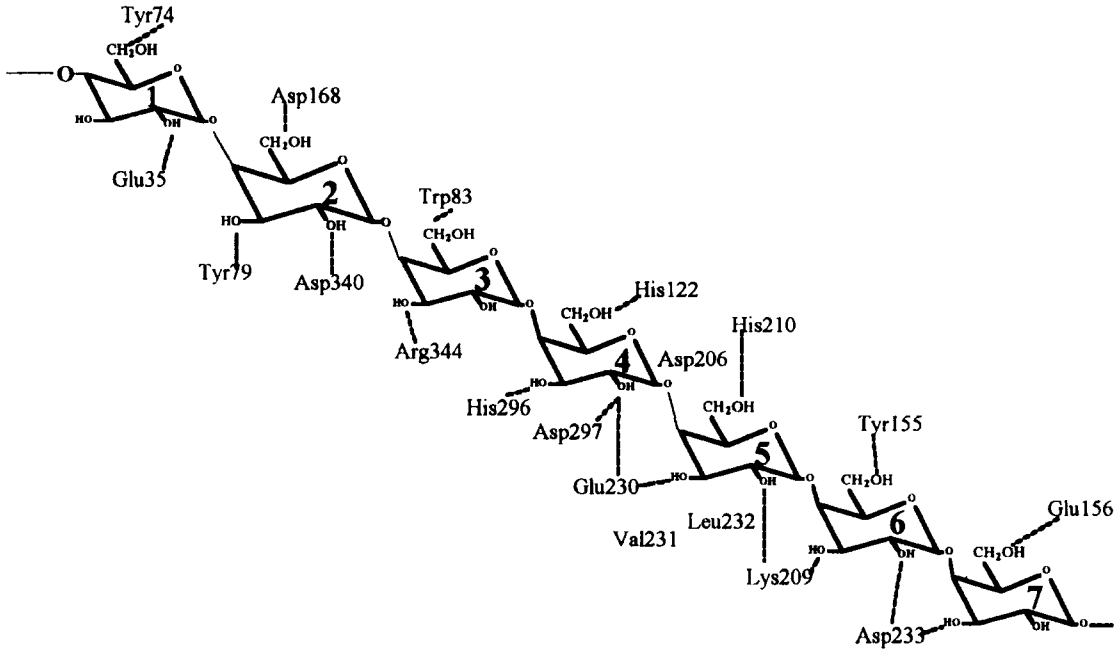

Fig. 2. Proposed substrate binding model of α-amylase and starch.

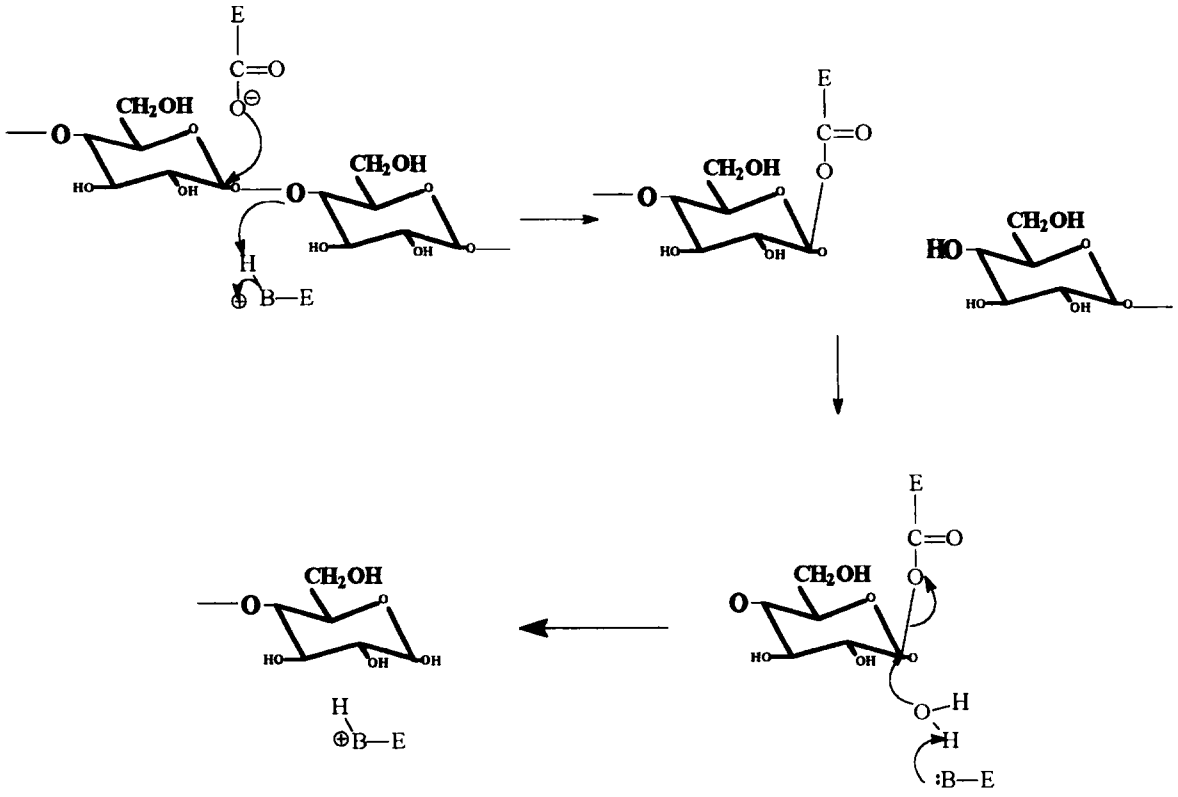

Fig. 3. Proposed nucleophilic double-displacement mechanism for α-amylase. E, enzyme; B, electrophilic catalytic group.

amylopectin, starch, and limit dextrins to give products without branch points. These enzymes hydrolyze pullulan to give maltotriose [7].

Amyloglucosidases are exoactive enzymes that can hydrolyze 1,4-, 1,6-, and 1,3-linkages although the relative rates of hydrolysis of 1,6- and 1,3-linkages are 15–30 times slower than that of 1,4-linkages [4]. When starch is treated with an amyloglucosidase, almost 90% of the weight of starch is converted to glucose.

EXPERIMENTAL

Materials

Waxy corn starch and octenyl succinic anhydride were donated from National Starch and Chemical Co. α-Amylase, amyloglucosidase, and pullulanase were obtained from Sigma Chemical Co. and were stored at below 0°C. All other chemicals were of reagent grade and were used as such.

Methods

The synthesis and degree of substitution (ds) determination have been described elsewhere [8].

Reducing Sugar Assay. The Nelson–Somogyi method [9] for reducing sugar assay were used here. The following solutions were used for this assay. For Solution A, 15 g of potassium tartrate and 30 g of anhydrous sodium carbonate were dissolved in 300 ml of water. Twenty grams of sodium bicarbonate was added to this solution. In another flask, 180 g of anhydrous sodium sulfate was dissolved in 500 ml of boiling water. These two solutions were then mixed and made up to 1 L with water.

Solution B was 5 g of copper sulfate pentahydrate and 45 g of sodium sulfate in 250 ml of water.

For solution C, 25 g of ammonium molybdate was dissolved in 450 ml of water, then 21 ml of concentrated sulfuric acid was added slowly, with stirring. Three grams of sodium arsenate heptahydrate was dissolved in 25 ml of water and added to the molybdate solution. This was then incubated for 24 h at 37°C and stored in a brown bottle.

For Solution D, 4 ml of concentrated sulfuric acid was added to 100 ml of water to form 0.75 *M* sulfuric acid.

Just before use, 500 ml of Solution A and 125 ml of Solution B were mixed to form Solution E and 100 ml of Solution D was mixed with 50 ml of Solution C to form Soluton F.

One milliliter of the solution with the degraded substrates was mixed with 1 ml of Solution E. This was heated at 100°C for 15 min and then cooled rapidly to room temperature. One milliliter of Solution F was then added and mixed thoroughly. Three milliliters of water was later added and the absorbance was measured at 520 nm.

Glucose Assay. Glucose assay was carried out using the D-glucose assay kit from Boehringer–Mannheim Corporation. Here, the D-glucose was phosphorylated to glucose-6-phosphate in the presence of the enzyme hexokinase and adenosine-5′-triphosphate, with the simultaneous formation of adenosine-5′-diphosphate. This was followed by the oxidation of glucose-6-phosphate by nicotinamide-adenine dinucleotide phosphate (NADP) in the presence of the enzyme glucose-6-phosphate dehydrogenase. During this reaction, gluconate-6-phosphate and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) were formed. The amount of NADPH formed was equal to the amount of D-glucose present in the medium and this was measured spectrophotometrically by absorbance at 365 nm.

Complete degradation to glucose was attempted by following the procedure reported by Kennedy *et al.* [7]. This method utilizes a multiple-enzyme regime of α-amylase, amyloglucosidase, and pullulanase. Enzymatic activity is defined as the amount of enzyme required to produce 1 mg/ml of dextrose equivalent in 1 min from 5 ml of 1% soluble starch at 60°C and pH 5.0. The dextrose equivalent was measured using the DNS assay with maltose standards.

One hundred twenty-five milligrams of the substrate was dispersed in 1 ml of ethanol. Ten milliliters of 1 *M* NaOH was added slowly while thoroughly mixing with a vortex mixer. After complete addition of the base, further mixing was effected for a minute to ensure complete dissolution. Ten milliliters of deionized water was then added and further mixing was effected for 1 more min. The pH was brought to 5.0 using glacial acetic acid, then 0.125 U of α-amylase, 22.5 U of amyloglucosidase, and 0.625 U of pullulanase were added to the solutions. Hydrolysis was carried out for the appropriate time period with continuous stirring at 60°C. The enzyme regimes were then deactivated by bringing the solution to a boil. This was then subjected to degradation assay.

RESULTS AND DISCUSSION

The degradation of starch completely to glucose was carried out following the procedure laid out by Kennedy *et al.* [9]. The procedure involves the utilization of α-amylase, amyloglucosidase, and pullulanase. The rationale behind this is that while α-amylase and amyloglucosidase are capable of hydrolyzing 1,4-glucosidic linkages, pullulanases are capable of hydrolyzing 1,6-linkages. Different combinations of these enzymes were tried and the optimum amounts of these enzymes were arrived at by Kennedy *et al.* Under the conditions of hydrolysis described, almost 99.5%, by weight, of starch from any source was hydrolyzed into glucose.

The activities of the enzymes were found to be 363, 367, and 578 U/mg for α-amylase, amyloglucosidase, and pullulanase, respectively. Hydrolysis was conducted at pH 5.0 and 60°C for appropriate amounts of time. It was found that starch was completely degraded within the first hour as determined by the reducing sugar assay.

Initially, the much more popular dinitrosalicylic acid assay for determining reducing sugar was used. But it was found that the substituents altered the absorption intensities. This was confirmed by synthesizing the maltose OSA derivative at different ds levels. This showed that beyond a molar ratio level of 0.01, OSA increased the absorption intensities twofold compared to maltose at the same concentration. Hence, this method was discarded and the Nelson–Somogyi method [8] was used for all reducing sugar assays, which proved to be fairly consistent with or without the substituent.

As expected, the extent of degradation is inversely proportional to the ds (Fig. 4). Even at the very low ds of 0.03, the extent of degradation is only about 79%. The extent of degradation for 0.07 OSA was about 63%, while that for 0.11 OSA was 58%.

The degradation of 0.03 and 0.07 ds OSA starch proceeded without any lag time. But the rates of degradation were different for both substrates. In fact, for 0.03 OSA, a single slope for the plot between extent of degradation and time can be seen. But for 0.07 ds OSA starch, almost two slopes can be seen: a lower one at lesser degradation times and a sharp increase at longer degradation times before leveling off. This is also seen in the case of 0.11 ds OSA except that, in this case, there is almost a lag time at low degradation times, indicating very low degradation extents. Then the degradation rate increases before leveling off.

This double slope indicates the presence of two different species, and the actions of enzymes on these substrates are different. The glucose assay provides some idea as to the nature of the species present in the medium. It can be seen that the amount of glucose present after 2 h of degradation is very low even for 0.03 ds OSA (Fig. 5). Yet at this point, a considerable amount of degradation has taken place. At 3 h, the amount of glucose has risen quite considerably. This observation is made for the higher-ds OSA starches too, except that the times are 1 h longer. This suggests that degradation takes place in stages whereby the enzymes produce several limit dextrins with the substituent. As soon as there is one chain with no substituent on it, the enzymes are capable of converting it to glucose very quickly. This

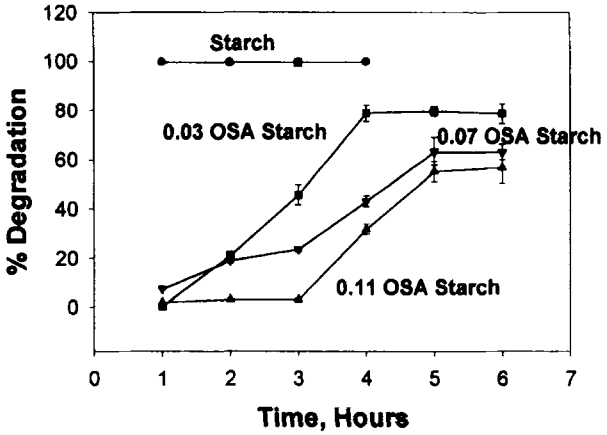

Fig. 4. Percentage degradation by the multienzyme regime as a function of time as followed by the Nelson–Somogyi method for reducing sugar assay.

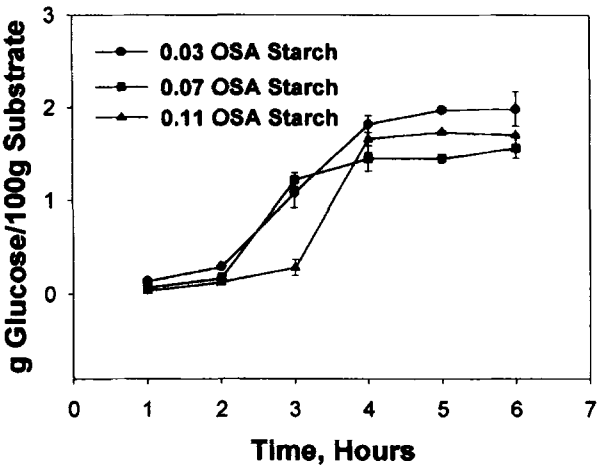

Fig. 5. Amount of glucose produced by enzymic hydrolysis as a function of time.

is the situation seen after 3 h of degradation. Beyond this point, the amount of glucose remains the same, and so does the extent of degradation. This shows that the presence of a small amount of substituent poses a great hindrance to the enzymes for degradation. It should be noted that the amount of glucose at the end of 6 h of degradation is only 1.5–2.5%, by weight, of the original material used. The amount of glucose from unmodified starch could not be measured, as the reading on the instrument went off the scale.

CONCLUSIONS

The multiple-enzyme regime was a very effective method of degrading starch to glucose in a relatively short period of time. In this work, starch was completely degraded as shown by the reducing sugar assay technique. The substituted starches, on the other hand, showed a greater resistance to degradation and this resistance was directly proportional to the ds of the sample. The correlation between the extent of degradation and the amount of glucose in the mixture shows that the presence of substituents in the chains restricts the activity of the enzymes. Several limit dextrins were produced by the enzymes during the initial stages of degradation, which were then rapidly degraded to glucose moieties. This was shown by the sudden increase in the amount of glucose in the reaction mixture.

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