NOVEL MODIFICATION OF CORNSTARCH BY IMMOBILIZED α-AMYLASE

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ABSTRACT

Limited hydrolyses of cornstarch in 2 or 10% dispersions with resin-bound α-amylase (*Bacillus subtilis*) provided modified starches of high molecular weight in yields up to 93%, with a significantly reduced tendency to retrograde. Because of the exoenzymic nature of the hydrolysis, degrees of polymerization of the byproduct oligosaccharides were confined to the range of 1–8. Properties of the enzymatically modified cornstarches, such as viscosity-concentration relationships, iodine-staining characteristics, solution stability, and molecular weight, were studied. The results suggest that the amylose component was hydrolyzed preferentially.

The stability of aqueous starch dispersions often determines their acceptance in many industrial and food applications. The extent of retrogradation of starch pastes is related inversely to temperature and directly to concentration. At room temperature, gelling or precipitation may occur readily in cornstarch dispersions of moderate (eg, 2%) concentration. Amylose, the linear molecular component of starch, is chiefly responsible for this phenomenon. Consequently, starches that consist exclusively of branched amylopectin, such as those derived from waxy varieties of corn or sorghum grain, have been used when amylose crystallization, either during or after an application, is a disadvantage. The more costly waxy starches differ in rheologic properties from the ordinary types, with amylose content in the range of 17–28%. Alternatives for preparing stable amylaceous dispersions have included use of amylopectin obtained by starch fractionation, and chemical derivatives and modifications of starch (eg, hydroxyethyl ether and oxidized starches) that minimize amylose retrogradation by blocking of hydrogen-bonding sites and by codispersion of starches with fatty materials that complex with amylose.

[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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The purpose of this study was to prepare an enzyme-modified starch with improved solution stability. We were prompted to do this study because we observed unusual results during hydrolysis of starch fractions with α-amylase bound to a phenol-formaldehyde resin. These results showed that the hydrolysis rate for amylose by the bound enzyme was much greater than for amylopectin. In contrast, soluble α-amylase hydrolyzed the two starch fractions at roughly the same rate (1). This discrimination between structural varieties apparently depends on steric factors that involve both the macromolecular substrate and the porous polymeric enzyme carrier. A selective enzymic degradation of the amylose component of whole starch thereby would yield a product having properties approaching those of the amylopectin fraction. The extent to which the outer branches of amylopectin are hydrolyzed was not determined in our previous work (1) because of the presence of residual amylose impurities. Little or no hydrolysis occurred with the bound enzyme in one experiment using waxy maize starch as substrate. We now have completed a limited study of the hydrolysis of whole cornstarch dispersions by this system and describe procedures used for the hydrolysis with immobilized α-amylase and some properties of the modified polysaccharides obtained.

During the cooking of starches, the amylose component is leeched from the swelling granules preferentially. Therefore, in dispersions having incomplete disruption of the starch granules, a bound enzyme is expected to act exclusively on the amylose-rich dispersed phase. Preferential hydrolysis based on such selective solubilization presumably would occur regardless of the choice of the insoluble enzyme carrier.

MATERIALS AND METHODS

Crystalline α-amylase (E.C. 3.2.1.1) from *Bacillus subtilus* was purchased from the Sigma Chemical Co., St. Louis, MO.

Cornstarch was Globe 3005, a commercial grade of pearl starch made by CPC International, Inc., Englewood Cliffs, NJ. The starch, which was not defatted, contained 10.6% moisture, 0.04% nitrogen, and 0.06% ash; intrinsic viscosities (25°C) were 1.75 and 1.44 in 1*N* KOH and 90% dimethyl sulfoxide (DMSO) (10% water), respectively.

Waxy maize starch used as a control in viscosity-concentration studies was Amioca from American Maize-Products Co., Hammond, IN. Moisture content was 17.7% and intrinsic viscosity in 90% DMSO was 1.44. Steam-jet cooked waxy maize, 11% slurry, had an intrinsic viscosity of 0.95.

The enzyme carrier was Duolite S-761, a phenol-formaldehyde adsorbent resin in particle form (passes 40-mesh, retained on 60-mesh screen) (Diamond Shamrock, Process Chemicals Div., Morristown, NJ). The first step of the enzyme attachment procedure consisted of a 3-hr adsorption process at room temperature in which 200 g (70 g db) of resin pretreated by a wash with 0.1*M* acetate buffer solution, pH 5, was suspended in 400 ml of the same buffer that contained 910 mg of soluble α-amylase. Adsorption of 97.4% of enzyme was recorded. In the second step, the sorbed enzyme was fixed on the resin by treatment of the drained sorption complex with 400 ml of a 1.25% glutaraldehyde solution for 90 min at room temperature. The enzyme-resin complex was washed

with 3 L of the pH 5 acetate buffer and with 8 L of 0.1% soluble starch solution to remove any unbound enzyme. The complex had an effective activity of 0.6μM apparent maltose per minute per gram.

Starches were cooked in a continuous steam-jet cooker (Penick and Ford, Ltd., Lyndhurst, NJ) to achieve substrate concentrations up to 10% that would be dispersed effectively yet not gel or retrograde during treatment with the enzyme at 40° C. High-pressure steam produced momentary temperatures of about 163° C and considerable hydraulic shear force in the orifice. The resultant reduction in intrinsic viscosity of the cornstarch, which was dependent on the cooking concentration, was from 1.44 to 0.94 dl/g at 4.2% concentration and to 1.14 dl/g at 19.5%. Starch slurries in distilled water at either 4.2 or 19.5% solids were gelatinized in the "hydroheater" at 163° C and then cooled to about 95° C after expansion in the flash chamber. Steam pressure to the cooker was 125 psi and the back pressure in the region past the hydroheater was 100 psi. Dispersions were diluted to either 2 or 10% concentrations and maintained for about 15 min at a temperature of about 70° C until just before treatment with the enzymes.

The immobilized α-amylase digests of the cornstarch substrates were made batchwise in either Fernbach or Erlenmeyer flasks, while suspension of the insoluble enzyme complex was maintained by agitating in a shaking water bath at 40° C. The enzyme-resin complex/starch substrate ratio was approximately 1:1 on a dry solids basis. The α-amylase action was stopped in all cases by filtering off the insoluble complex with a coarse, sintered-glass filter. Isolation of the modified starches was initiated immediately after the filtration.

Enzyme-treated starch dispersions were poured slowly into a Waring Blendor (high-speed setting) that contained sufficient absolute methyl alcohol to result in a final alcohol concentration of 80% by volume. The modified starch precipitates were recovered on a Buchner funnel with Whatman No. 54 filter paper. The precipitates were washed in absolute methyl alcohol, vacuum pressed under a rubber dam on the funnel, dried overnight in a vacuum desiccator over $CaCl_2$, and equilibrated at 23° C and 50% relative humidity.

The 80% methyl alcohol solubles were concentrated in vacuo at 50° C until the methyl alcohol essentially was removed and the aqueous solutions were acidified, pH 2–3, with HCl and extracted three times with equal volumes of chloroform to remove fatty materials. The 80% alcohol-soluble materials were then recovered by freeze-drying.

Reducing powers of the immobilized enzyme digests were determined on a Technicon AutoAnalyzer by Procedure A of Robyt et al (2), using maltose as a standard.

The color intensity of the iodine complexes in enzymic digests was measured essentially by the method of McCready and Hassid (3). One hundred microliters of 0.2% I_2 in a 2.0% KI solution were added to the samples, which were then diluted to 10 ml with water. The absorbancies of the solutions at 590 nm were determined with a Gilford 300-N spectrophotometer.

For spectral-transmission curves of the iodine complexes of isolated starches, dry samples, about 50 mg, were weighed into 50-ml volumetric flasks and 20 ml of 90% DMSO (10% water) were added. The samples were dissolved with a minimum of heating, cooled, and diluted with water. All subsequent dilutions were with water. Typically, 1 ml of dispersed sample in 90% DMSO was transferred to a 50-ml volumetric flask; 10 ml of 1% potassium acid tartrate

solution was added, followed by about 30 ml of water, 1 ml of I_2 solution (0.2 g of I_2 and 2 g of KI/100 ml), and sufficient water to a final volume of 50 ml. The solutions were stored in the dark for 30 min before measuring the spectral transmission with a Beckman DB recording spectrophotometer. Appropriate blanks containing DMSO were used.

Molecular size distributions of the modified starches were determined with a Waters Ana-Prep gel permeation chromatograph on two columns (122 cm × 9.5 mm ID) of Waters Hydrogels II and VI that have molecular weight separation ranges for dextran of 500–10,000 and 1,000–2,000,000, respectively. The columns were calibrated with Sephadex Dextrans T10, T20, T40, T110, T150, T500, and α-methyl glucoside. Starch components, however, are unlikely to elute at the same volume as dextrans of identical molecular weights, because amylose is sorbed to a small degree by Hydrogel and the branched structures of amylopectin materials occupy smaller hydrodynamic volumes than do linear dextrans of identical molecular weights. Operating conditions were: solvent, water; sample concentration, 0.08%; sample size, 2 ml; flow-rate, 1 ml/min; oven temperature, 45°C. The samples were filtered through porous glass (0.4–1.4-μm pore size) before their injection into the columns.

Oligosaccharides, produced by the action of immobilized α-amylase on the starch substrates, were recovered in the 80% methyl alcohol solubles. Aqueous solutions of the oligosaccharides (500 μg/5–10 μl) were analyzed by high-pressure liquid chromatography (HPLC) on a μBondapak NH_2 column in a

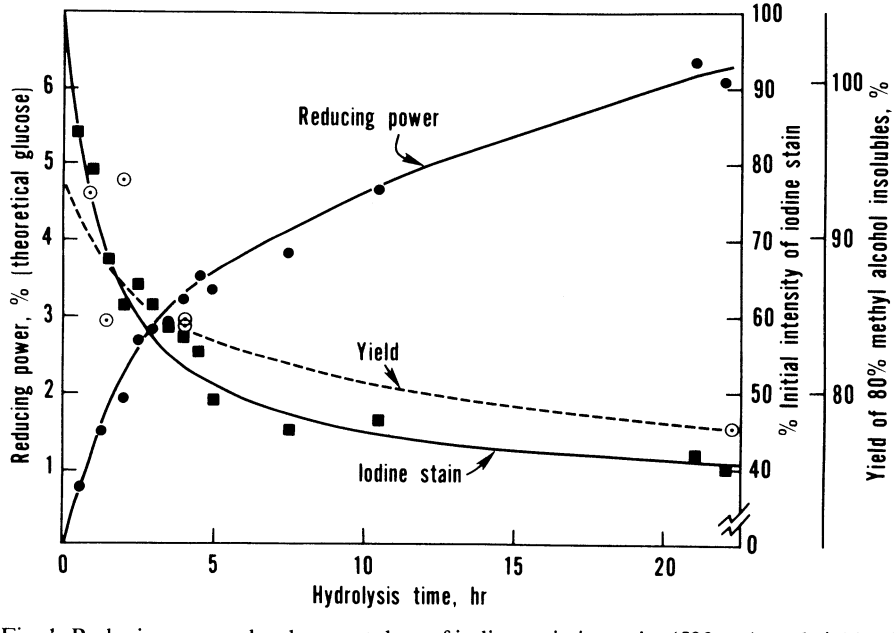

Fig. 1. Reducing power development, loss of iodine stain intensity (590 nm), and yields of 80% methyl alcohol-insoluble products as affected by time of treatment of 2% starch substrate with bound α-amylase at 40°C. Average yield of three untreated control starch substrates was 94.6%.

Waters Associates ALC-100 chromatography unit. Separation was effected in 7:3 acetonitrile/water at a constant flow rate of 2 ml/min. Elution positions were calibrated with maltooligosaccharide standards that ranged from D-glucose to maltononose.

The viscosity-concentration relationships for the modified starches were determined with a Ferranti-Shirley cone-plate viscometer (Ferranti Electric Company, Inc.) at 25° C. Viscosity measurements were begun as soon as possible after dispersion in water with heating as required and followed by rapid cooling to 25° C. The samples were subjected to shear rates from 0 to 1,000 rpm and developed within 10 sec; the viscosity was calculated from the recorded rheograms. Viscosity was measured again on all samples after standing for 24 hr at room temperature.

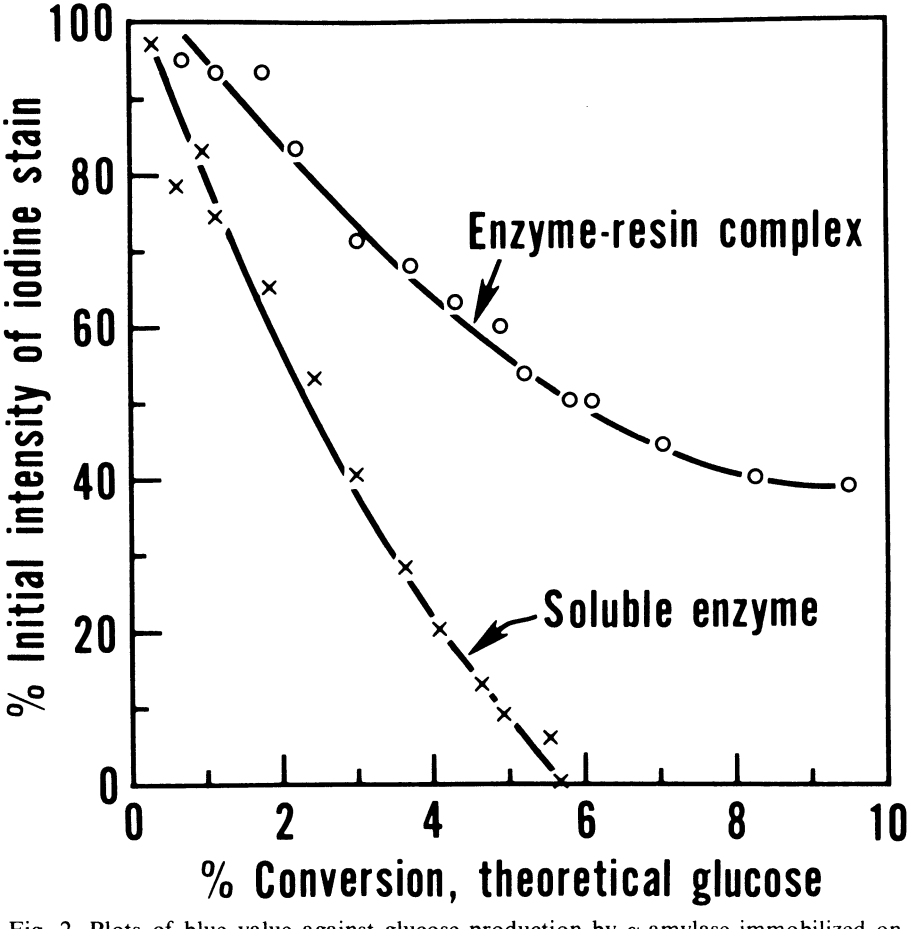

Fig. 2. Plots of blue value against glucose production by α-amylase immobilized on phenol-formaldehyde resin (O) and soluble α-amylase (X). Amylase levels are equal on an activity basis for both experiments. Substrate was 1% soluble starch.

Intrinsic viscosity measurements of starches in 90% DMSO solution or 1*N* KOH were made at 25 ± 0.05°C in a size 100 Cannon-Ubbelohde capillary viscometer.

RESULTS AND DISCUSSION

The development of practical substrate concentrations considerably above the 0.08% used in our initial work (1) was a first concern. Therefore, 5% slurries of corn and tapioca starches were cooked at 90° C for 1.5 hr with moderate stirring and diluted to 2% for reaction with the enzyme-resin complex. Significant modification of these starch substrates occurred within 2 hr. Polysaccharides of the hydrolysate isolated from the oligosaccharides by methyl alcohol precipitation displayed substantial decreases both in retrograde tendencies of their aqueous solutions and in color intensities of their iodine complexes.

Based on these encouraging results, further work was undertaken to 1) establish techniques and practical conditions for dispersion, reaction, and recovery, 2) characterize both the high-molecular weight residue and oligosaccharide byproducts, and 3) investigate resin-substrate interactions. The practical aspects were emphasized by using commercial cornstarch exclusively and increasing substrate concentration to 10% in several preparations.

The change in the α-amylase activity pattern from endoenzymic to exoenzymic on immobilization results not only in markedly different molecular size species in the hydrolysates but also in a more rapid rate of amylose digestion than that of amylopectin. To determine if the resin, without enzyme, also may adsorb amylose preferentially, a starch dispersion was treated with the resin under conditions identical to those used with the resin-enzyme complex. The treatment totaled 6 hr, with the resin being replaced by fresh resin after the first 3-hr contact. The resin-treated starch was recovered after precipitation from 80% methyl alcohol. Its iodine stain color was reduced in intensity by 11%; a 4% aqueous dispersion had slightly greater clarity and stability than that of the control starch. A 6% dispersion of this material, however, underwent phase separation 10 min after cooling. Even though the resin alone adsorbed some amylose as evidenced by the reduced iodine-staining capacity and the increased clarity, the amylose content is still sufficient to cause gelling. Throughout this article, the term "control" refers to steam-jet cooked starches that were precipitated from an 80% methyl alcohol solution.

Enzyme Hydrolysis

Reaction mixtures contained 5 g of enzyme-resin complex (0.6 units/g) for each 100 ml of a 2% dispersion of cornstarch at pH 5. The rate of enzymic conversion at 40° C was followed by the changes in reducing power and the iodine stain intensity at 590 nm of the digest. Figure 1 illustrates the progress of the reaction for a 2% substrate concentration. Curves for both reducing power and iodine stain indicate marked slowing of reaction after about 2–3 hr. At the time when reducing power reaches about 6% of theoretical glucose available in the substrate, the intensity of the iodine color has decreased to approximately 40% of the original iodine absorbance value. Soluble α-amylase digests show no iodine color when they attain the same reducing value (Fig. 2). Data for Fig. 2 were obtained on a 1% starch substrate.

Reaction progress was also observed by determining yields of the enzyme-modified starch products. Yields of 80% alcohol-insoluble material (Fig. 2) decline rapidly during the first 2–4 hr, level off thereafter, and parallel loss of iodine color as depicted in Fig. 1. The average recovery for three control starches was 94.6%.

Modified Starch Properties

Intrinsic Viscosity. Intrinsic viscosities of the methyl alcohol-precipitated starches were not significantly different from those of the control substrates (0.99 dl/g), except for the 22-hr hydrolysis product, which was lower (0.85 dl/g).

Solution Properties. Solution stability of the precipitated starches was determined quantitatively by measuring changes in relative viscosities over six days of 6% dispersions held at 25°C in a capillary viscometer (Fig. 3). Viscometric stability is directly related to the extent of hydrolysis. Initial relative viscosity of the 22-hr sample of modified starch is lower than the others, in agreement with its relatively lower intrinsic viscosity. The 22-hr sample was also dispersed at a concentration of 8% and its relative viscosity of 27.9 remained stable during the 13 days it was monitored. At 12% concentration, however, the 22-hr sample formed a soft gel within several hours, with some slight solid-phase separation. Heating to boiling restored the original clarity.

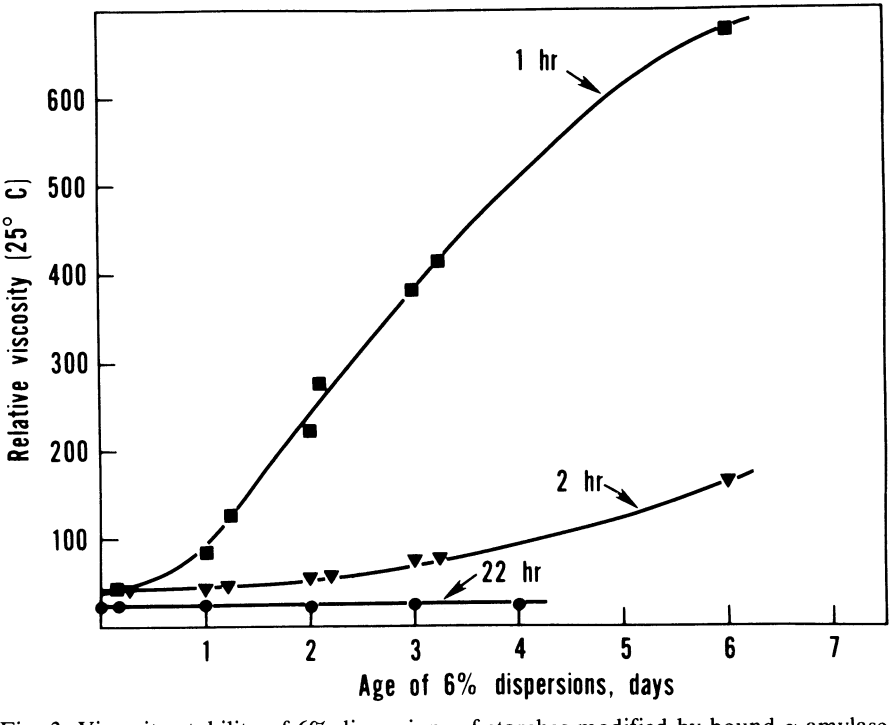

Fig. 3. Viscosity stability of 6% dispersions of starches modified by bound α-amylase, measured in a low-shear capillary viscometer at 25°C.

Light transmission of the dispersions was also measured, because it reflects changes such as increased molecular association that can lead to precipitation. Figure 4 shows changes at room temperature in optical densities (550 nm) of 8% dispersions of a control and of the 2- and 22-hr bound enzyme-treated starches. Initially, the three samples had similar clarities, but the unmodified control developed turbidity rapidly and gelled 10 min after cooling. This opaque, hard gel could not be liquefied by either shearing or heating. Almost all of the total increase in turbidity that occurred in the 2-hr sample took place within the first few days. After several days, the 2-hr sample set to a soft gel that could be liquefied easily by stirring. Turbidity increases in the 22-hr sample were relatively small.

The viscosity-concentration relationships for the modified cornstarches were determined at 25°C in a Ferranti-Shirley cone-plate viscometer. The viscosity behavior was non-Newtonian and shear sensitive. Comparison of the 1- and 22-hr samples with steam-jet cooked waxy maize shows similarities (Table I). The viscosity measurements were repeated after 24-hr storage at approximately 23°C. Some setback occurred in the enzyme-modified starches at the higher concentrations; however, no setback was observed in dispersions of the steam-jet cooked waxy maize. Much higher concentrations of the modified starches probably could not have been made without some retrogradation. The control starches gelled rapidly and irreversibly at 4% concentration.

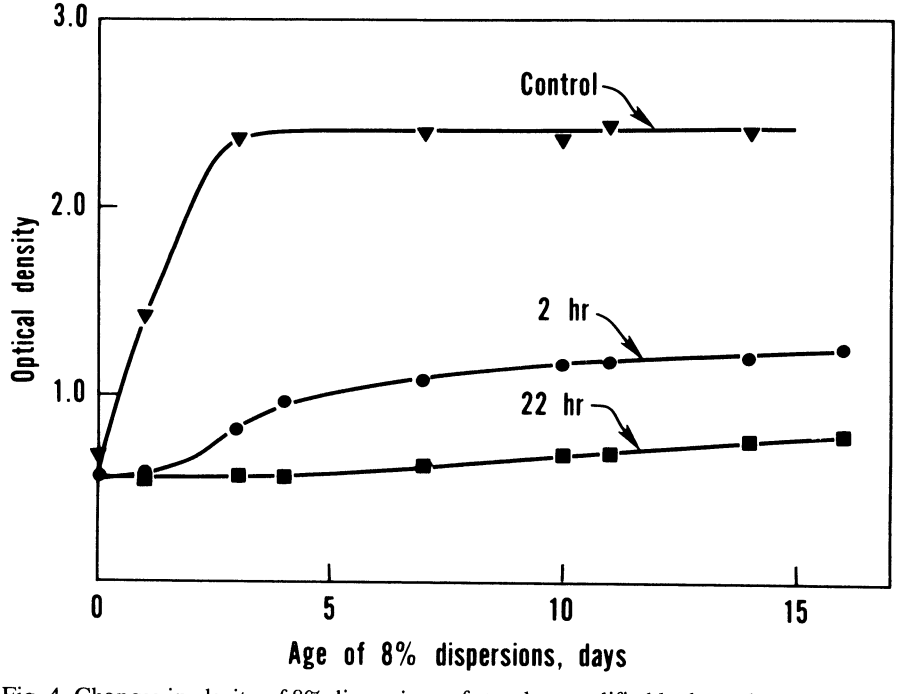

Fig. 4. Changes in clarity of 8% dispersions of starches modified by bound α-amylase on storage at room temperature.

Starch-Iodine Complex. The progressive decrease in intensity of the starch-iodine color (Fig. 1) is attributed chiefly to the degradation of amylose, although some loss of intensity would occur also if outer branch segments of amylopectin were hydrolyzed. Therefore, transmission spectra were obtained (Fig. 5) of the iodine complexes of the enzymatically modified starches that were isolated by alcohol precipitation. The curves, which compare equal concentrations of the modified starch-iodine complexes, show the decrease in the extinction coefficients with increased hydrolysis time. A modest downward shift in the wavelength of maximum absorbance occurs from 620 nm for unmodified starch to 607–609 nm for the hydrolyzed product. Bailey and Whelan (4) have shown that amylose chains of DP 300 and above retrograde rapidly. The λ maximum for the iodine color of these amylose molecules is 645 nm. These authors further show that amylose chains of 20–80 glucose units have a λ maximum of 600–610 nm. These shorter amylose chains are stable in solution. A reasonable assumption is that the starch digest obtained by α-amylase immobilized on phenol formaldehyde contains relatively unaltered amylopectin plus short chains of amylose of less than 100 glucose units. Such starch solutions would be expected to retrograde either slowly or not at all.

Molecular Weights of Products

Molecular weight distribution in several of the immobilized enzyme digests (2% substrate) was analyzed by gel permeation chromatography (Fig. 6). Little or no material of intermediate molecular weight was evident even after 22 hr of hydrolysis, at which time about 22% of the hydrolysis products were soluble in 80% methyl alcohol. In contrast, we showed in previous work that soluble enzyme acting on an amylose substrate gave a wide distribution of product weights, whereas bound enzyme hydrolysates of comparable reducing power had a distribution like that in Fig. 6 (1). Digests of 10% starch concentrations gave similar results.

TABLE I
Twenty-Four–Hour Setback in Enzyme-Treated Cornstarch Dispersions Held at Room Temperature

	Viscosity (cps)							
	Concentration of Paste							
	6%		8%		10.9%		12%	
Shear Rate	Initial	24 hr	Initial	24 hr	Initial	24 hr	Initial	24 hr
				1-hr Enzyme Treatment				
100 rpm	51	52	68	113	186	255[a]	...	...
1,000 rpm	18	17	22	28	68	78[a]	...	...
				22-hr Enzyme Treatment				
100 rpm	37	37	47	71	...	...	220	355[a]
1,000 rpm	13	13	17	23	...	...	79	99[a]
				Unmodified Waxy Maize Starch				
100 rpm	248	216	329	336	...	...	...	...
1,000 rpm	41	37	77	77	...	...	...	...

[a]Heated to boiling and then cooled before viscosity measurement at 25°C in Ferranti-Shirley cone-plate viscometer.

The distribution of oligosaccharides in the immobilized enzyme digest was determined by HPLC (Table II). Data of Robyt and French (5) for the amylolysis of amylose and amylopectin with free enzyme from *B. subtilis* are included for comparison. Only traces of oligosaccharides of a degree of polymerization greater than 8 could be detected in the chromatographs of the bound enzyme

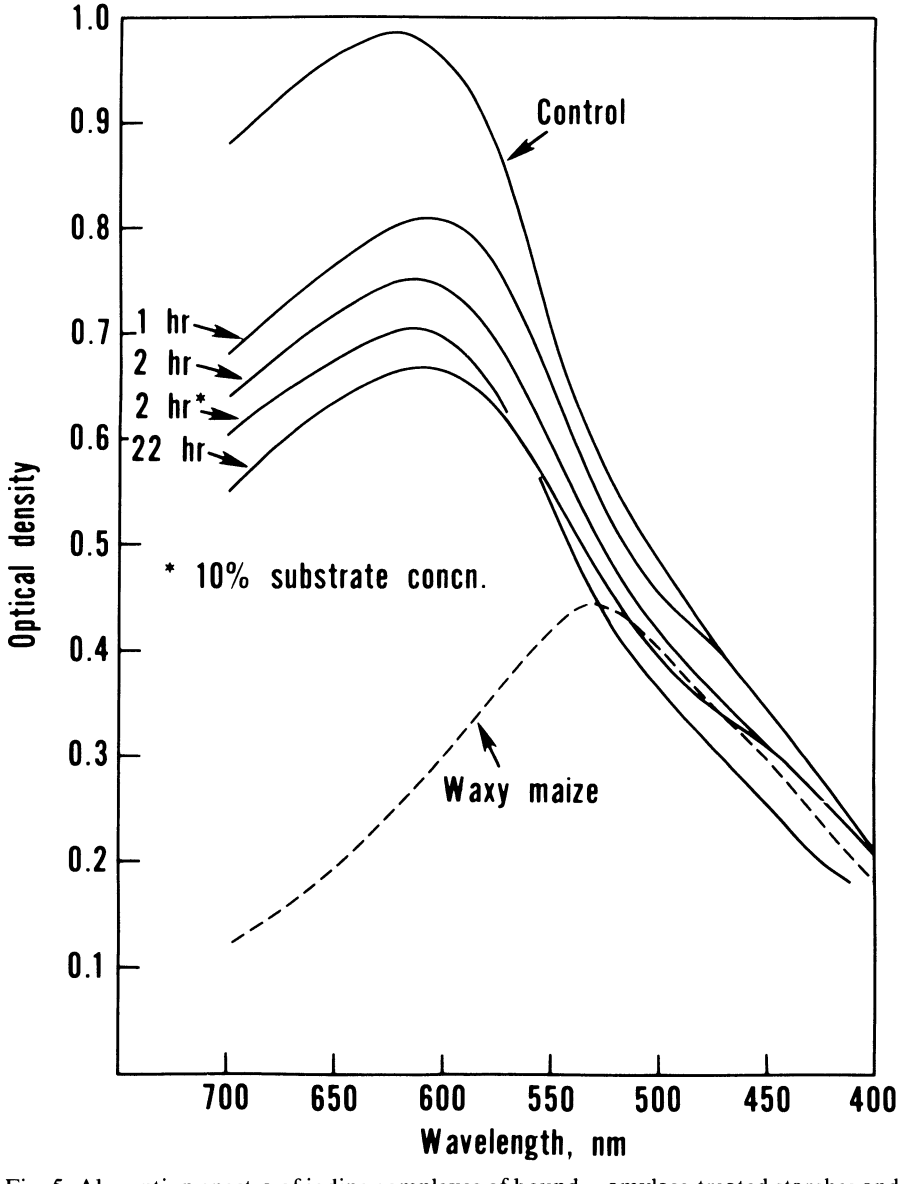

Fig. 5. Absorption spectra of iodine complexes of bound α-amylase-treated starches and control starches.

digests. Yields of oligosaccharides in the 2-hr hydrolysate that retains 79.4% of initial blue color correspond to those obtained at the achroic point of amylose hydrolysis with free enzyme. The oligosaccharide composition from 10% starch digests were similar to those recorded in Table II.

CONCLUSIONS

Cornstarch has been modified by partial hydrolysis with α-amylase immobilized on a phenol-formaldehyde resin in which preferential hydrolysis of the amylose component appears to have occurred. Evidence supporting a selective hydrolysis of the amylose fraction includes 1) the reducing power-iodine stain relationships observed during the reaction, 2) oligosaccharide distribution in degradation products in agreement with the distribution pattern for amylose hydrolyzed by free α-amylase, and 3) changes in properties of the isolated modified starches, such as a decrease in the extinction coefficient of the starch-iodine complexes and the improvement in clarity and stability of aqueous dispersions.

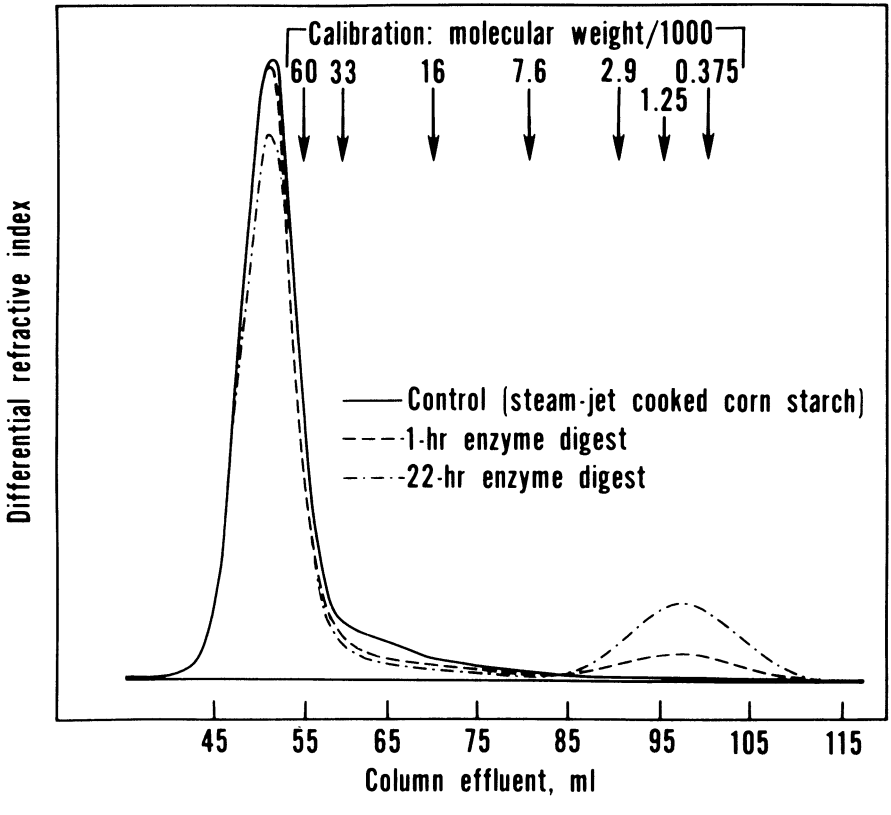

Fig. 6. Gel permeation chromatograph of whole digests treated with bound α-amylase for 1 and 22 hr showing changes in molecular-weight distribution of products. Molecular weight was calibrated with dextran standards.

TABLE II
Percentage Composition of Oligosaccharides Produced by α-Amylolysis

	Immobilized α-Amylase[a]		Free α-Amylase[b]			
	Cornstarch		Amylose		Amylopectin	
Sugar	2 hr	22 hr	1 hr	3 hr	1 hr	3 hr
G_1	2 (3)	9	2	5	1	3
G_2	16 (23)	16	10	12	6	8
G_3	22 (21)	24	13	22	8	11
G_4	11 (9)	10	6	11	1	3
G_5	16 (19)	29	10	15	5	7
G_6	27 (22)	12	21	30	14	27
G_7	6 (2)	0.6	15	5	10	9
Over G_7	0 (Trace)	0	23	0	55	32

[a]Values are percent of total high-pressure liquid chromatography peak areas obtained with 2% substrate concentrations; 10% substrate concentration values are given in parentheses.
[b]Data of Robyt and French (5) with *Bacillus subtilis* α-amylase, expressed as "weight %," all sugars to G_7 are linear. Three-hour amylose digest is at achroic point.

Those amylose molecules having molecular weights in the range at which retrogradation occurs most readily are speculated to be preferentially sorbed onto the resin. If the resin preferentially adsorbs amylose, these sorbed molecules are likely the ones on which the bound enzyme acted, thereby clearing the resin surface for continued reaction. Although the process is incomplete insofar as removal or hydrolysis of all amylose or both are concerned, the molecular species of amylose most prone to association and retrogradation apparently have been removed selectively.

The bound-enzyme reactions were accomplished by procedures that have good potential for scaled-up production. The industrial applications for which these modified starches seem suitable include gel-resistant adhesives and food preparations. The extent of any enzyme action on the amylopectin fraction of whole starch was not determined, and this could be a crucial factor in the performance of these modified starches in bakery products, particularly with respect to staling properties.

Acknowledgment

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