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Significant differences in the activities of α-amylases in the absence and presence of polyethylene glycol assayed on eight starches solubilized by two methods

Rupendra Mukerjea, Giles Slocum, Romila Mukerjea and John F. Robyt*

Laboratory of Carbohydrate Chemistry and Enzymology, Department of Biochemistry, Biophysics, and Molecular Biology, 4252 Molecular Biology Building, Iowa State University, Ames, IA 50011, USA

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Abstract—Starch is a reserve chemical source of the energy of the sun found in plants as a water-insoluble granule that differs in their chemical and physical properties, depending on the source. The granules can be solubilized by heating in water or by treatment with various reagents, such as 1 M NaOH. α-Amylases are widely distributed enzymes that initiate the hydrolysis of starch into low molecular weight maltodextrins. We recently found that the activities of a single α-amylase on two different starches were significantly different. We then determined the activities of *Bacillus amyloliquefaciens* and porcine pancreas α-amylases, using eight different starches, solubilized by two methods: autoclaving at 121 °C and 1 M NaOH at 20 °C. There were significant differences in the activities of both of the amylases on all eight of the starches. Previously, it had been found that polyethylene glycol (PEG) stabilized and activated the activities of both enzymes, using a soluble amylose as the substrate. Addition of PEG to the enzymes greatly increased the activities on the eight starches, but the activities still differed significantly. The different activities with the starches were hypothesized as differences in the amounts of secondary and tertiary structures that are partially retained when the different starches are solubilized; the activities on addition of PEG is hypothesized as the formation of highly active species from a series of less active forms.

Keywords: *Bacillus amyloliquefaciens*; Porcine pancreas; α-Amylases; Assays; Eight starches; Secondary starch structure; Tertiary starch structure; Polyethylene glycol; Enzyme activations

1. Introduction

Starch is a reserve chemical form of the energy of the sun found in green leaves, stems, tubers, cereal grains, seeds, nuts, and fruits of plants. Many kinds of starches provide the major source of food energy in the diets of humans and are increasingly used industrially as bio-renewable materials for the formation of a diverse array of products, for example, ethanol, high-fructose syrups, cyclomaltodextrins, maltodextrins, adhesives, paper and textile sizing agents, and so forth. Starch consists of two types of polysaccharides, amylose, a linear α-(1→4)-glucan, and amylopectin, a branched α-(1→4)-glucan with 5% α-(1→6)-branch linkages.[1] The two polysaccharides are found in 15–30% and 85–70%, respectively, with the exception of some mutants, such as amylomaize-VII starch that is 70% amylose and 30% amylopectin and waxy maize starch that is 100% amylopectin.[1] The glucans are combined in a water-insoluble granule, whose size, shape, chemical and physical properties differ, depending on the source of the starch.[1,2] The water-insoluble granules can be solubilized by heating in water to 100 °C or by autoclaving at 121 °C or by a cold chemical treatment with 1 M NaOH or with 85:15 (v/v) Me_2SO–H_2O at 20 °C.[3–5]

α-Amylases [EC 3.2.1.1] are widely distributed enzymes formed by bacteria, fungi, plants, and animals

* Corresponding author. Tel.: +1 515 294 1964; fax: +1 515 294 0453; e-mail: jrobyt@iastate.edu

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that hydrolyze the α-(1→4)-glucosidic bonds of starch into maltodextrins of varying sizes, depending on the particular enzyme.[6] α-Amylases are customarily assayed by using commercial Lintner's soluble starch.[6–8] The most accurate assay method is the measurement of the linear increase in the reducing value over a period of time at a concentration of starch that gives the maximum velocity.[8] Several methods are available for the measurement of the increase in the reducing value, such as the alkaline copper method,[7,9] the alkaline ferricyanide method,[10] and the micro copper bicinchoninate method,[8] but not the alkaline 3,5-dinitrosalicylic acid (DNSA) method, which is very insensitive and not quantitative due to over oxidation.[9]

We recently found, however, that the assay of *Bacillus amyloliquefaciens* α-amylase on solubilized potato starch and waxy maize starch gave two significantly different assay values. The assays were repeated, and the same result was obtained, within experimental error. Because α-amylases are commonly assayed, using Lintner's soluble starch, it has apparently been assumed that the assay obtained on soluble starch would be the same as an assay on *any* solubilized starch. Because this seems to be a reasonable assumption, the result that we obtained was a surprise. In the present study, we have assayed *B. amyloliquefaciens* α-amylase on eight, relatively common, starches from different sources, with different physical and chemical properties. The starches were solubilized by two methods, and we used two different α-amylases to show that the differences observed in the assays were not peculiar to *B. amyloliquefaciens* α-amylase.

We also had recently obtained an interesting result in which five different α-amylases were both stabilized and significantly activated by the addition of polyethylene glycols (PEGs) of specific molecular sizes.[11] Because these results are important in that the study and use of enzymes are greatly facilitated if the enzymes are stable and have the highest maximum activity possible, we have assayed the two α-amylases in the absence and presence of their specific PEGs, using the eight starches as substrates.

The eight starches were chosen for (a) their being a major food starch (e.g., potato, rice, wheat, and maize starches), (b) their ease of gelatinization (e.g., potato, shoti, and tapioca starches), (c) their unusual composition of amylose and amylopectin (e.g., amylomaize-VII starch with 70% amylose and waxy maize starch with 100% amylopectin), (d) their differences in their type of X-ray patterns (e.g., A-type for maize, wheat, rice, and waxy maize starches; B-type for potato, amylomaize-VII, and shoti starches; and C-type for tapioca starch), and (e) the somewhat exotic character of shoti starch with its unusual flat-plate granule morphology and its relatively wide use in Asia as a therapeutic agent for stomach and intestinal problems.

2. Experimental

2.1. Materials

2.1.1. Starches. Maize, potato, rice, waxy maize, amylomaize-VII, wheat, and shoti starches were freshly prepared from their various mature sources, using standard procedures,[12] but without the addition of sodium bisulfite. Mature maize seeds were obtained from Dr. Thomas Binder of Archer, Daniel, Midland in Decatur, IL. Mature amylomaize-VII and waxy maize seeds and tapioca starch were obtained from National Starch and Chemical Co., Bridgewater, NJ. Mature rice and potatoes were obtained from a local market. Shoti starch was isolated from mature *Curcuma zedoaria* tubers obtained from Dr. M. Kitaoka, National Food Research Institute, Tsukuba, Japan.

2.1.2. Enzymes. *B. amyloliquefaciens* α-amylase (HT concentrate) was obtained from Miles Laboratories (Elkhart, IN), and porcine pancreatic α-amylase was a crystalline enzyme obtained from Sigma Chemical Co. (St. Louis, MO), catalogue No. A6255.

2.1.3. Chemicals. 2,2′-Bicinchoninate was obtained from Sigma Chemical Co., and the polyethylene glycols with average molecular weights of 1.0 and 1.5 kDa were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of the highest grade possible.

2.2. Methods

2.2.1. Solubilization of the starches. The starch granules were solubilized by suspending 110 mg in 7 mL of water and autoclaving them at 121 °C for 30 min; after cooling, 1 mL of 400 mM imidazole·HCl buffer (pH 6.5), containing 10 mM $CaCl_2$, was added, followed by dilution with water to 10 mL. The solutions were centrifuged at 10,000*g* for 10 min, and the supernatants were used for the assays. The second method used involved the stirring of 110 mg of granules in 1 mL of 1 M NaOH for 15 h at 20 °C, dilution with water to 6 mL, neutralization with 1 M HCl, and the addition of 1.0 mL of 400 mM imidazole·HCl buffer (pH 6.5), containing 10 mM $CaCl_2$, followed by dilution with water to 10.0 mL.

2.2.2. Use of the enzymes. The enzymes were diluted from the stock solutions with 40 mM imidazole·HCl buffer (pH 6.5), containing 1 mM $CaCl_2$, and 0.04% (w/v) PEGs as wanted, PEG 1.0K for BAA and PEG 1.5K for PPA, which previously had been found to give the maximum degree of activation for each enzyme.[11] The diluted enzyme for each starch reaction was added to the starch solutions in 20-s intervals. A single enzyme

dilution was used for the triplicate assays of a single starch, with and without PEG for the two methods of solubilizing the starch (12 assays). Any one-enzyme dilution was not allowed to stand any longer than 2 min before being added to the starch solutions.

2.2.3. Assays of the α-amylases. The activities of BAA and PPA were determined by the measurement of the increase in the reducing value of the maltodextrin products of the reaction. The reducing value was determined by the micro copper bicinchoninate method, using maltose as the standard.[13] A plot of the microgram of apparent maltose versus time (min) was prepared, and the slope of the linear part of the curve was determined and converted into micromoles of glucosidic bonds hydrolyzed per minute by dividing by 342, the molecular weight of maltose. This was the unit of activity (U) and was actually the maximum velocity of the reactions, because the concentrations of the starches were saturating above 1 mg/mL (unpublished data), and the concentrations of the starches ranged from 3 to 9 mg/mL.

For the assays, 100 μL of properly diluted enzyme, to give a linear line from 0 to 30 min, within the range of the reducing value method used in the assay, was added to 1.90 mL of the starch solutions that were preincubated at 37 °C for 10 min; 100 μL aliquots were taken every 5 min for 30 min and added to 900 μL of 0.01 M NaOH to stop the reaction. Dilutions were made when necessary, and the reducing value was measured in triplicate by the micro copper bicinchoninate method, using maltose as the standard.[13] Triplicate assays were made for four of the starches for both enzymes, in the absence and presence of PEGs. The assays for all eight of the starches were completed on two consecutive days within 5 h each. The data for each reaction was plotted (microgram of bond hydrolyzed vs minute of reaction), and the slope of the straight line was converted to micromole of linkages hydrolyzed per minute and used as the assay value.

3. Results

3.1. Activities for BAA and PPA

The activities for BAA and PPA on the eight starches solubilized by autoclaving and by 1 M NaOH, both in the absence and presence of adding PEG 1.0K to BAA and PEG 1.5K to PPA are given in Tables 1 and 2, respectively.

3.2. Reaction of BAA with starches solubilized by autoclaving

The activities on the autoclaved starches without PEG were identical for shoti and waxy maize starches and were the highest for this assay condition. The activity on tapioca starch was less by 8.2%; on rice starch, it was less by 10.1%; on potato starch, it was less by 18.3%; on maize starch, it was less by 21.0%; on wheat starch, it was less by 51.0%; and on amylomaize-VII starch, it was less by 72.3% than the two highest starches from shoti and waxy maize that gave the highest activities.

3.3. Addition of PEG 1.0K to BAA and its reaction with starches solubilized by autoclaving

On adding PEG 1.0K to BAA and assaying it on the autoclaved starches, all of the activities increased from a low value of 28.7% for waxy maize starch to a high of 150.0% for wheat starch. Other increases were 29.7% for shoti starch, 49.5% for tapioca starch, 51.1% for potato starch, 89.3% for maize starch, and 123.0% for amylomaize-VII starch.

3.4. Reaction of BAA with starches solubilized by 1 M NaOH

When the 1 M NaOH solubilized starches were assayed without the addition of PEG to BAA, five of the starches

Table 1. Activity of *Bacillus amyloliquefaciens* α-amylase in the absence and presence of polyethylene glycol 1.0K with eight starches solubilized by autoclaving at 121 °C for 20 min and by 1 M NaOH at 20 °C for 15 h

Starches	Method of starch solubilization			
	Autoclaved (121 °C, 30 min)		1 M NaOH, 20 °C, 15 h	
	w/o PEG 1.0K U[a]/mL ± SD[b]	w/ PEG 1.0K U[a]/mL ± SD[b]	w/o PEG 1.0K U[a]/mL ± SD[b]	w/ PEG 1.0K U[a]/mL ± SD[b]
Shoti	317 ± 8	411 ± 8	241 ± 3	802 ± 4
Waxy maize	317 ± 8	408 ± 9	272 ± 5	593 ± 7
Tapioca	293 ± 4	438 ± 6	471 ± 9	1103 ± 8
Rice	288 ± 5	444 ± 7	251 ± 4	455 ± 6
Potato	268 ± 7	405 ± 7	397 ± 3	822 ± 4
Maize	262 ± 5	496 ± 7	291 ± 4	355 ± 4
Wheat	210 ± 3	524 ± 11	292 ± 6	501 ± 4
Amylomaize-VII	184 ± 4	411 ± 4	233 ± 4	404 ± 4

[a] U = 1.0 μmol of α-(1→4)-bonds hydrolyzed per minute.
[b] SD = standard deviation.

Table 2. Activity of porcine pancreatic α-amylase in the absence and presence of polyethylene glycol 1.5K with eight starches solubilized by autoclaving at 121 °C for 30 min and by 1 M NaOH for 15 h

Starches	Method of starch solubilization			
	Autoclaved (121 °C, 30 min)		1 M NaOH, 20 °C, 15 h	
	w/o PEG 1.5K U[a]/mL ± SD[b]	w/ PEG 1.5K U[a]/mL ± SD[b]	w/o PEG 1.5K U[a]/mL ± SD[b]	w/ PEG 1.5K U[a]/mL ± SD[b]
Maize	640 ± 7	905 ± 7	442 ± 6	824 ± 54
Wheat	626 ± 19	1003 ± 21	435 ± 7	864 ± 26
Shoti	613 ± 16	3137 ± 6	1219 ± 12	3462 ± 47
Waxy maize	557 ± 1	2500 ± 26	1141 ± 6	1330 ± 6
Amylomaize-VII	523 ± 8	856 ± 30	455 ± 4	858 ± 7
Rice	451 ± 6	798 ± 21	417 ± 23	890 ± 7
Tapioca	281 ± 3	2841 ± 24	1007 ± 9	2450 ± 2
Potato	262 ± 2	2100 ± 1	1274 ± 20	3152 ± 68

[a] U = 1.0 μmol of α-(1→4)-bonds hydrolyzed per minute.
[b] SD = standard deviation.

(from tapioca, potato, maize, wheat, and amylomaize-VII) had higher activities for BAA than the autoclaved starches without the addition of PEG to the enzyme. The activity on tapioca starch increased 60.8% over the autoclaved starch; activity on potato starch increased 48.1%; activity on wheat starch increased 39.0%; activity on amylomaize-VII starch increased 26.6%; and the activity on maize starch increased 11.1%. For three of the starches (from shoti, waxy maize, and rice), the activities for BAA were lower for the starches solubilized by 1 M NaOH than they were for the activities for the starches solubilized by autoclaving. The activity on shoti starch was less by 24.0%; the activity on waxy maize was less by 14.2%; and the activity on rice starch was less by 12.8%.

3.5. Addition of PEG 1.0K to BAA and its reaction with starches solubilized by 1 M NaOH

When PEG 1.0K was added to BAA and it was assayed on the 1 M NaOH solubilized starch, five of the starches (from shoti, waxy maize, tapioca, potato, and rice) gave higher activities than the autoclaved starches. The BAA activity on tapioca starch was the highest with a 151.8% over the activity obtained from the autoclaved tapioca starch; the activity on potato starch increased 103.0%; the activity on shoti starch increased 95.1%; the activity on waxy maize starch increased 45.3%; and the activity on rice starch increased 2.5%.

3.6. Assay of PPA with starches solubilized by autoclaving

The activities for PPA on the eight starches solubilized by autoclaving and by 1 M NaOH, both in the absence and presence of adding PEG 1.5K to PPA are given in Table 2. The activities for PPA on the autoclaved starches without PEG, ranged from a high of 640 U/mL on maize starch to a low of 262 U/mL on potato starch. The activity on wheat starch was less than the activity on maize by 2.2%; activity on shoti starch was less by 4.2%; the activity on waxy maize starch was less by 13.0%; the activity on amylomaize-VII starch was less by 18.3%; the activity on rice starch was less by 29.5%; the activity on tapioca starch was less by 56.1%; and the activity on potato starch was less by 59.1%.

3.7. Addition of PEG 1.5K to PPA and its reaction with starches solubilized by autoclaving

On adding PEG 1.5K to PPA and determining the activity on the starches solubilized by autoclaving, the activities were greatly increased for all of the starches from a low of 41.4% for maize starch to a high of 911.0% for tapioca starch and increases of 60.2% for wheat starch, 63.7% for amylomaize-VII starch, 76.9% for rice starch, 348.8% for waxy maize starch, 411.8% for shoti starch, and 701.5% for potato starch.

3.8. Reaction of PPA with starches solubilized by 1 M NaOH

When the starches were solubilized by 1 M NaOH and PEG was not added to PPA, four of the starches (from shoti, waxy maize, tapioca, and potato) gave higher activities than the autoclaved starches in which PEG was not added to PPA. The activity on potato starch increased the most by 386.3%; activity on tapioca starch increased 258.4%; activity on waxy maize starch increased 104.8%; and activity on shoti starch increased 98.9%. There were also four starches (from maize, wheat, amylomaize-VII, and rice) where the activities were lower than the autoclaved starches: 44.8%, 43.9%, 15.0%, and 8.2%, respectively.

3.9. Addition of PEG 1.5K to PPA and its reaction with starches solubilized by 1 M NaOH

When PEG 1.5K was added to PPA and it was assayed on the 1 M NaOH solubilized starches, there was an

increased activity on three of the starches from shoti, rice, and potato of 10.4%, 11.5%, and 50.1%, respectively. The highest activity obtained in the study was 3462 ± 47 U/mL on shoti starch. Four of the starches (from maize, rice, wheat, and waxy maize) gave lower activities of 9.8%, 11.5%, 16.0%, and 88.0%, respectively, than the starches solubilized by autoclaving and adding PEG 1.5K to PPA. There was one starch, amylomaize-VII, where there was no change in the activity when PEG 1.5K was added to PPA and the activity was determined on the starch solubilized by the two methods.

4. Discussion

The results show that the assays of the two α-amylases on the eight starches were significantly different when the starches were solubilized by autoclaving. Changing the method of solubilization to a cold chemical method, using 1 M NaOH, changed the assay values, increasing them for some of the starches and decreasing them for others. The addition of specific sizes of PEGs to the enzymes gave greatly increased activities over the reactions where the PEGs were not added. The fact that both enzymes showed the same phenomenon of having significantly different activities on the eight solubilized starches shows that the phenomenon was not specific for a single, particular α-amylase.

The activities of the two α-amylases, however, differed, but they should not be compared with each other, in that these α-amylases have different product specificities and produce different sized products due to a different number of D-glucose binding subsites at the active site, nine for BAA and five for PPA.[6,14,15] What should be compared are the activities of the individual enzymes, acting on the different starches, both in the absence and in the presence of PEG.

The differences that have been observed in the activities on the different starches suggest that they are due to differences in the structures of the individual starches. It is recognized that starch granules from different sources have different amounts of molecular and supramolecular order that is produced by secondary structures (helices and double helices of α-(1→4)-linked starch chains) and tertiary structures (inter- and intramolecular associations through hydrogen and hydrophobic bonding).[2,16,17] There is a wide diversity exhibited by starch granules from different sources in their sizes and shapes,[2,18] composition of amylose and amylopectin,[1,2,16,17] degrees of crystallinity and types of X-ray diffraction patterns,[2,16,17] and in their susceptibility toward enzyme hydrolysis.[19–21] Further, it has been shown that α-amylases will not hydrolyze the glucosidic linkages of D-glucopyranose units involved in double helices and in retrograded starch.[22]

It was thus hypothesized that the differences observed in the activities of a single kind of α-amylase assayed on the eight starches was due to the retention in solution of varying amounts of the secondary and tertiary structures found in the starch granules. This hypothesis was strengthened when it was found that the two methods of solubilization gave differences in the activities, indicating that the mechanism of solubilization by the two methods gave differences in the amounts of the secondary and tertiary structures that were retained in solution.

Some investigators have hypothesized that differences in the gelatinization temperatures and susceptibilities of the starch granules to enzyme digestibility are due to differences in the fine structure of amylopectin, especially the relative abundance of unit-chains with an approximate degree of polymerization of 8–12 and 16–26.[23] The relative abundance of the unit-chain lengths of amylopectin may be a contributing factor, but it is only in as much as the chain lengths contribute to the secondary and tertiary structures in the granules in solution. Another possibly important contribution to these structures is the differences in the distribution of branch linkages and the clustering of the branches in the amylopectin molecules in the granules and in solution.[24]

A comparison of the maximum activities of the two enzymes in the presence of PEG was made for the eight starches that were solubilized in 1 M NaOH (see Table 3). The order of the first three starches was the same for the two enzymes, but in reverse order: tapioca, potato, and shoti starches for BAA and shoti, potato, and tapioca starches for PPA. Waxy maize starch was fourth for

Table 3. Comparison of the maximum activities of *Bacillus amyloliquefaciens* and porcine pancreatic α-amylases in the presence of PEG on the eight starches solubilized by 1 M NaOH

Order	Starches	U[c]/mL
Bacillus amyloliquefaciens α-amylase[a]		
1	Tapioca	1103
2	Potato	822
3	Shoti	802
4	Waxy maize	593
5	Wheat	501
6	Rice	455
7	Amylomaize-VII	404
8	Maize	355
Porcine pancreatic α-amylase[b]		
1	Shoti	3462
2	Potato	3152
3	Tapioca	2450
4	Waxy maize	1330
5	Rice	890
6	Wheat	864
7	Amylomaize-VII	858
8	Maize	824

[a] *Bacillus amyloliquefaciens* α-amylase with PEG 1.0K.
[b] Porcine pancreatic α-amylase with PEG 1.5K.
[c] One unit (U) = 1.0 μmol of α-(1→4)-glycosidic bonds hydrolyzed per minute.

both enzymes. Wheat and rice starches were fifth and sixth for BAA and PPA, but in reverse order. The last two starches, amylomaize-VII and maize had the lowest activities in the same order for the two enzymes.

The dramatic effects of adding PEG to the two enzymes, especially when comparing the differences in the two methods of solubilization for certain starches, such as the 252% increase for BAA on tapioca starch, 203% increase for BAA on potato starch, and the 195% increase for BAA on shoti starch; and the 150% increase for PPA on potato starch, and the 110% increase for PPA on shoti starch, and 112% increase for PPA on rice starch is striking. Because of these large increases in activity on adding PEG to the enzymes, it is tempting to think that the PEGs are having an affect on the secondary and tertiary structures of the solubilized starches. The PEG, however, is only effective when it is added to the enzyme at a concentration of 0.04% (w/v) before being added to the starches. Even so, if the PEG would be free in the enzyme digest, it would be at a very low concentration of 0.002% (w/v) or 20 μg/mL, and it is highly unlikely the PEG is affecting the structures of the solubilized starches whose concentrations ranged from 3 to 9 mg/mL, depending on the starch. Further, in our initial study of the activation and stabilization of BAA and PPA by PEG, when the PEG was only added to the substrate (soluble amylose), there was no activation or stabilization of the enzymes observed, thus, indicating that the activation effect of PEG was exclusively the interaction with the enzymes.[11]

Our hypothesis on the effects of PEG on the activation of the enzymes is that the enzymes in dilute solution have several structural forms in equilibrium with each other and these forms have different activities. The addition of 0.04% (w/v) PEGs to the enzymes gives a single optimal structure in which the enzyme is stabilized and has the maximum enzyme activity.[11] We also speculated that the PEGs might be increasing the degree of multiple attack, but the percent blue value versus the reducing value curves for the enzymes in the absence and presence of PEG were identical (unpublished data), indicating that the degree of multiple attack was not being altered.[25]

In conclusion, the study definitively shows that a single kind of α-amylase, acting on starches from different sources had widely and significantly different activities. These differences are hypothesized to be due to differences in the amounts of secondary and tertiary structures that are retained from the structures of the starch granules when in solution. The amount of structure that is retained is dependent on the structures in the starch granules and the method of solubilizing the granules. The addition of 0.04% (w/v) PEG to the enzyme greatly enhances the activity of the enzyme, but there still are significant differences in the activities observed for the starches from different sources. The differences observed for the action of a single α-amylase on the starches are thus an indication of the differences of the degrees of secondary and tertiary structures that remain when the starches are solubilized.

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