ELSEVIER

Carbohydrate Research 337 (2002) 1113–1117

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

Reactions of alpha amylases with starch granules in aqueous suspension giving products in solution and in a minimum amount of water giving products inside the granule

Cheol Yook, John F. Robyt*

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

Received 24 September 2001; accepted 5 April 2002

Abstract

Porcine pancreatic alpha amylase (PPA) and *Bacillus amyloliquefaciens* alpha amylase (BAA) were allowed to react with starch granules from maize, waxy maize, amylomaize-7, and potato in an aqueous suspension with a starch to water ratio of 1:10 and in a minimum of water with a starch to water ratio of 1:1. Quantitative amounts of the maltodextrin products were determined by TLC and scanning densitometry. The two alpha amylases gave different products that were characteristic of their unique action patterns. The percent conversion differed for the different kinds of starches and for the two kinds of reaction conditions. Maize and waxy maize starches were converted into about twice as much maltodextrins than were amylomaize-7 and potato starches by both enzymes and under both reaction conditions. The aqueous suspension gave much greater conversion into maltodextrins than did the minimum water condition. BAA gave 3–14% greater conversion of the granules into maltodextrins than did PPA, with the exception of potato starch.

Keywords: Alpha amylases; porcine pancreatic alpha amylase; *Bacillus amyloliquefaciens* alpha amylase; starch granules; reaction in aqueous suspension; reaction in minimum water; maltodextrins

1. Introduction

Starch granules from different botanical sources have different sizes, shapes, and physical properties.[1] In general, starch granules are less susceptible to amylase hydrolysis than gelatinized starch,[2] and at one time it was believed that whole starch granules were resistant to hydrolysis by amylase. There have been reports that have shown alpha amylases digest whole starch granules with differences in the kinds of starch granules[3] and differences in the source of alpha amylase.[4]

Kimura and Robyt[2] have shown that *Rhizopus niveus* glucoamylase (EC 3.2.1.3) degraded a wide variety of starch granules, although to a varying degree that was dependent on the botanical source of the starch. The starches divided into three groups: waxy maize starch granules were the most susceptible (98% converted to D-glucose); barley, maize, and tapioca starches were susceptible to an intermediate degree (having 82, 79, and 75% conversion, respectively); and amylomaize-7, shoti, and potato starches were the least susceptible (having 21, 15, and 13% conversion, respectively). The starches in the first two groups could be converted into 50% D-glucose, giving a classical, resistant, 'Swiss cheese' shell structures to the granules.

Glucoamylase is potentially capable of catalyzing the hydrolysis of both α-(1→4) and α-(1→6) glycosidic linkages of gelatinized starch, thereby giving 100% conversion of starch into D-glucose.[5] The hydrolysis of starch granules by glucoamylase is known to require a starch-binding domain that is distinct from the active site (the starch-hydrolyzing domain).[6–9] If the starch-binding domain is removed, glucoamylase ceases to be able to hydrolyze whole starch granules, but will still hydrolyze solubilized starch.[8,9] Starch granules will adsorb glucoamylase, which has the starch-binding domain, from solution in different proportions (33–62%), depending on the particular kind of starch but will not

* Corresponding author. Tel.: +1-515-294-1964; fax: +1-515-294-0453.

E-mail address: jrobyt@iastate.edu (J.F. Robyt).

0008-6215/02/$ - see front matter
PII: S0008-6215(02)00107-6

adsorb glucoamylase that is devoid of the starch-binding domain.[2]

Kim and Robyt[10] devised a method in which glucoamylase reacted with the starch granules to give 100% retention of the D-glucose product inside the granules. Waxy maize starch granules gave a maximum of 54% conversion into D-glucose; maize starch granules gave a maximum of 35% conversion; and amylomaize-7 starch granules gave a maximum of 24% conversion.

The action patterns of porcine pancreatic alpha amylase (PPA) and *Bacillus amyloliquefaciens* alpha amylase (BAA) have been shown to give distinct products from amylose, amylopectin, and maltodextrins.[11,12] PPA produced primarily maltose (G2), maltotriose (G3), and maltotetraose (G4);[11] and BAA produced primarily maltotriose (G3), maltohexaose (G6), and maltoheptaose (G7).[12] The formation of D-glucose was exclusively due to a secondary breakdown of the primary products and these alpha amylases produced only a very small amount of D-glucose.

In the present study, a comparison has been made of the action of the two diverse alpha amylases (EC 3.2.1.1) from porcine pancreas and from *B. amyloliquefaciens*, reacting with four kinds of starch granules from potato, waxy maize, maize, and amylomaize-7. Two types of reaction conditions of granules in aqueous suspension of 1:10 ratio of starch to water and a minimum of water of 1:1 ratio of starch to water.

2. Experimental

Materials.—Waxy maize, maize, and amylomaize-7 starches were obtained from Cerestar USA (Hammond, IN). Potato starch was obtained from National Starch and Chemical Co. (Bridgewater, NJ). Porcine pancreatic and *B. amyloliquefaciens* alpha amylases were obtained in crystalline form from Boehringer–Mannheim Corp. (Indianapolis, IN, USA). Whatman K5 silica gel TLC plates were obtained from Fisher Scientific (Chicago, IL).

Methods.—*Amylase assay*. Amylase (100 μL) was added to 1.9 mL of waxy maize starch solution (10.5 mg/mL) buffered with 50 mM imidazole–HCl (pH 6.5), containing 2 mM $CaCl_2$. Samples (100 μL) were taken 5, 10, 15, 20, and 30 min and added to **X** μL of 0.01 M NaOH [**X** can be from 100 to 900 μL, depending on the amount of dilution required]. After all of the samples are taken, 100 μL are added to 100 μL of the working reagent of copper bicinchoninate[13] in a microplate. A maltose standard curve is prepared by the addition of 100 μL of standard to 100 μL of copper bicinchoninate and the reducing value is measured by the copper bicinchoninate micro method.[13] The unit of activity (International Unit, IU) is defined as the number of μmoles of glycosidic bonds cleaved per min.

Amylase reaction in aqueous suspension. Each of the starches (100 mg) was suspended in 970 μL of 50 mM imidazole–HCl buffer (pH 6.5), containing 2 mM $CaCl_2$ and 0.02% w/v NaN_3. The ratio of starch to water was 1:10. Amylases (30 μL, 0.5–50 IU) were added to the starch slurry and mixed. The reaction was conducted at 37 °C, with occasional mixing. Samples (20 μL) were taken at various times, centrifuged, and the supernatants heated in a boiling water bath for 5 min to stop the reaction. The samples were then analyzed by quantitative TLC.

Amylase reaction in a low amount of water. Amylases (30 L, containing 50 IU) were added with mixing to 100 mg of potato, maize, waxy maize, and amylomaize-7 starches in 65, 70, 80, and 95 μL, respectively, of 50 mM imidazole–HCl buffer (pH 6.5), containing 2 mM $CaCl_2$, and 0.02% (w/v) NaN_3. The ratio of starch to water was 1:1. The starch–enzyme mixture was incubated at 37 °C in a closed vessel. The reaction was stopped at various times by the addition of 10 μL of 1 M HCl. The starches were dried in a stream of air (20 °C) for ~15 h. The reaction mixtures were analyzed by the addition of 905, 900, 890, and 875 μL of water to potato, maize, waxy maize, and amylomaize-7 starches, respectively, and allowed to stand 30 min at 20 °C and then centrifuged. The supernatants were analyzed by quantitative TLC.

Quantitative thin-layer chromatography. Samples (1–5 μL) were added to Whatman K5 silica gel (20 × 20 cm) plates. The plates were irrigated, using two ascents (18.5 cm path length each) of 85:20:50:50 MeCN–EtOAc–1-propanol–water.[14] The carbohydrates on the plates were visualized by dipping the plate into a methanolic solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4, followed by heating at 120 °C for 10 min.[15] The D-glucose and maltodextrins on the plate were quantitated, using an imaging densitometer (BioRad, model GS-670) with maltose standards (50–2000 ng).[14]

3. Results and discussion

The action patterns of PPA and BAA on amylose, amylopectin, and maltodextrins have previously been reported.[11,12] Maltose (G2), maltotriose (G3), and maltotetraose (G4) are known to be the primary products of the action of PPA.[11] The primary products of BAA are known to be G3, G6, and G7.[12] The present study shows that similar kinds of action patterns occur for the action of PPA and BAA with starch granules in aqueous suspension and in a minimum amount of water (Tables 1–3). Starch granules from four sources, maize, waxy maize, amylomaize-7, and potato were

studied. These starches were selected for their representative characteristics and properties: maize starch is a cereal grain starch that has 25% amylose and 75% amylopectin; waxy maize starch or high amylopectin starch has 100% amylopectin; amylomaize-7 starch or high amylose starch has 70% amylose and 30% amylopectin; and potato starch is a tuber starch with a large granule and 20% amylose and 80% amylopectin.

As the amount of PPA was increased in the aqueous suspension reaction (Table 1), D-glucose (G1) was formed and G2 was increased; G3 was slightly decreased and G4 was significantly decreased. Similarly, as the amount of BAA was increased, there was a decrease in the amounts of G6, and G7, with an increase in the amounts of G1, G2, G3, G5, and G8 (Table 1). These changes that occurred as the amount of enzyme was increased are due to the secondary hydrolysis of the primary products, G3 and G4 for the PPA reactions and G6, G7, and G8 for the BAA reactions.

At 50 IU PPA/g of starch granules, the reactions in the aqueous suspension and the reactions in a minimum amount of water gave primarily G1, G2, and G3 from all four types of starch granules at 12 and 120 h of reaction (Table 2). At 50 IU BAA/g of starch granules, the reactions in the aqueous suspension and the reactions in a minimum amount of water gave primarily G1–G8 from all four types of starch granules, with a predominance of G1–G5 from maize and waxy maize starch granules at 12 and 120 h of reaction (Table 3).

In the aqueous suspension reaction at 50 IU PPA/g of starch granules, maize and waxy maize starches were converted into 71.4 and 73.6%, respectively, G1–G4 after 120 h of reaction, and in the minimum amount of

Table 1
Relative percent composition of maltodextrin products from maize starch hydrolyzed by different enzyme units of porcine pancreatic alpha amylase (PPA) and *B. amyloliquefaciens* alpha amylase (BAA) after 24 h of reaction at 37 °C in an aqueous suspension of 1:10 ratio of starch to water

Enzyme	U/g starch	G1 [a]	G2	G3	G4	G5	G6	G7	G8	Total (%)
PPA	0.5	5.7	30.3	43.3	20.8	–	–	–	–	100
	5.0	10.2	41.8	42.1	5.9	–	–	–	–	100
	50	14.2	42.6	38.8	4.4	–	–	–	–	100
BAA	0.5	4.3	14.6	17.6	11.3	14.8	22.1	11.0	4.2	100
	5.0	9.4	15.9	17.3	10.1	20.6	17.8	5.2	3.8	100
	50	13.9	20.8	20.3	10.4	16.7	7.7	4.8	5.4	100

[a] G1, G2, G3, and so forth represent D-glucose, maltose, maltotriose.

Table 2
Weight percent of maltodextrin products from starch granules hydrolyzed by porcine pancreatic alpha amylase (50 IU/g of starch) at 37 °C for 12 and 120 h in an aqueous suspension and in a minimum amount of water

Reaction type	Starch	Time (h)	G1 [a]	G2	G3	G4	Total (%)
Aqueous suspension	Maize	12	3.70	14.20	13.60	0.54	32.0
Starch:water 1:10		120	11.93	34.03	23.53	1.91	71.4
	Waxy maize	12	4.16	13.84	14.44	0.89	33.3
		120	12.10	33.39	25.70	2.43	73.6
	Amylomaize-7	12	2.36	9.16	7.15	0.17	18.8
		120	9.42	19.51	8.50	1.02	38.4
	Potato	12	1.83	5.98	4.54	0.12	12.5
		120	8.66	16.75	6.44	0.51	32.4
Minimum water	Maize	12	1.76	4.50	3.92	0.27	10.4
Starch:water 1:1		120	3.78	8.25	4.08	0.48	16.6
	Waxy maize	12	1.42	4.38	4.00	0.21	10.0
		120	3.43	8.86	4.24	0.31	16.8
	Amylomaize-7	12	1.30	3.98	2.55	0.13	8.0
		120	3.80	8.67	2.28	0.03	14.8
	Potato	12	0.93	3.25	2.22	0.05	6.4
		120	2.20	5.90	1.77	0.00	9.9

[a] G1, G2, G3, and so forth represent D-glucose, maltose, maltotriose.

Table 3
Weight percent of maltodextrin products from starch granules hydrolyzed by *B. amyloliquefaciens* alpha amylase (50 U/g of starch) at 37 °C for 12 and 120 h in an aqueous suspension and in a minimum amount of water

Reaction type	Starch	Time (h)	G1 [a]	G2	G3	G4	G5	G6	G7	G8	Total (%)
Aqueous suspension Starch:water 1:10	Maize	12	3.33	4.61	4.90	2.59	5.17	2.49	1.13	1.27	25.5
		120	13.86	23.23	22.07	7.70	6.33	2.74	3.96	4.20	84.1
	Waxy maize	12	2.98	4.18	4.35	2.50	5.41	2.55	1.45	1.76	25.2
		120	13.39	21.38	19.69	6.76	6.57	2.28	4.65	5.20	79.9
	Amylomaize-7	12	2.05	3.21	3.60	1.85	3.26	1.11	0.50	0.52	16.1
		120	7.91	12.38	11.21	4.16	3.03	1.05	1.28	1.45	42.5
	Potato	12	1.40	2.06	2.12	1.18	2.09	0.66	0.41	0.40	10.3
		120	4.78	7.32	6.50	1.79	1.20	0.42	0.88	1.34	24.2
Minimum water Starch: water 1:1	Maize	12	2.00	3.43	3.06	1.25	1.49	0.77	0.55	0.51	13.0
		120	4.63	7.95	5.90	1.61	0.80	1.04	0.98	0.58	23.5
	Waxy maize	12	1.89	3.22	2.95	1.24	1.35	0.84	0.84	1.15	13.5
		120	4.19	7.13	5.26	1.76	0.95	1.71	1.54	0.95	23.5
	Amylomaize-7	12	2.17	3.40	3.21	1.45	1.23	0.44	0.37	0.55	12.8
		120	4.38	7.33	4.59	1.24	0.69	0.29	0.26	0.04	18.8
	Potato	12	1.49	2.23	2.14	0.34	0.65	0.25	0.26	0.28	7.6
		120	2.60	4.54	3.10	0.42	0.39	0.36	0.35	0.07	11.8

[a] G1, G2, G3, and so forth represent D-glucose, maltose, maltotriose.

water reaction, maize and waxy maize were converted into 16.6 and 16.8%, respectively, G1–G4 after 120 h of reaction (Table 2); in the aqueous suspension reaction, 50 IU PPA/g of starch granules, amylomaize-7 and potato starches were converted into 38.4 and 32.4%, respectively, G1–G4 after 120 h of reaction, and in the minimum amount of water reaction, amylomaize-7 and potato starches were converted into 14.8 and 9.9%, respectively, G1–G4 after 120 h of reaction (Table 2).

In the aqueous suspension reaction at 50 IU BAA/g of starch granules, maize, and waxy maize starches were converted into 84.1 and 79.9%, respectively, G1–G8 after 120 h of reaction, and in the minimum amount of water reaction, maize and waxy maize were both converted to 23.5%, G1–G8, after 120 h of reaction (Table 3); in the aqueous suspension reaction, 50 IU BAA/g of starch granules, amylomaize-7 and potato starches were converted into 42.5 and 24.2%, respectively, G1–G8 after 120 h of reaction, and in the minimum amount of water reaction, amylomaize-7 and potato starches were converted into 18.8 and 11.8%, respectively, G1–G8 after 120 h of reaction (Table 3).

For both alpha amylases and both types of reaction conditions, aqueous suspension and minimum amount of water, the percent conversion to D-glucose and maltodextrins was higher for maize and waxy maize starch granules and significantly lower for amylomaize-7 and potato starch granules. BAA gave higher percent conversions in both types of reactions for three of the starches (maize, waxy maize, and amylomaize-7) than did PPA, with the exception of potato starch in which PPA gave a slightly higher conversion than did BAA (Tables 2 and 3). Both alpha amylases gave the retention of the shape and morphology of the granules for all four types of starches, indicating that the reaction was taking place inside the granule. In the reaction in the aqueous suspension, the products diffused into the aqueous supernatant, but in the reaction in the minimum amount of water, a ratio of 1:1 of starch and water, the products were retained inside the granule. These products readily diffused when the granules containing the products were suspended in an aqueous solution.

The differences in the action patterns of PPA and BAA are due to differences in the number of glucose-binding subsites at the active site of the two enzymes. PPA was shown to have a relatively small binding site of five glucose subsites[11] and BAA was shown to have a binding site of nine glucose subsites, nearly twice that of PPA.[12] BAA has the catalytic site located between subsites 3 and 4, giving three subsites to the right and six subsites to the left of the catalytic site. PPA has the catalytic site located between subsites 2 and 3, giving two subsites to the right and three subsites to the left. In addition, BAA was proposed to have a dual product specificity in which after the initial cleavage of the α-(1$\rightarrow$4) linkage, either the chain to the right of the catalytic site diffuses away and the remaining chain bound to the enzyme diffuses into the three open binding sites to give G3 as a product, or the chain to the left of the catalytic site diffuses away and the remaining chain bound to the enzyme diffuses into the six open binding sites to give G6, G7, or G8 as products.[12] PPA, on the other hand, has five binding sites

with the catalytic site located between subsites 2 and 3, with two subsites to the right and three subsites to the left of the catalytic site.[11] It has been shown that only the chain to the right diffuses away after the initial cleavage and the remaining chain to the left diffuses to fill the two open binding subsites to give G2, G3, and G4 as products in a multiple attack pattern.[16]

In conclusion, both PPA and BAA will catalyzed the hydrolysis of starch granules to maltodextrins that were characteristic of their unique action patterns. Two reaction conditions were used, granules in an aqueous suspension with a starch to water ratio of 1:10 and in a minimum amount of water with a starch to water ratio of 1:1. Reactions in the aqueous suspension gave a much higher degree of conversion to 71–84% maltodextrins than reactions in the minimum amount of water that gave a relatively low degree of conversion to 10–24% conversion of the granules to maltodextrins. BAA gave 3–14% greater conversion of the granules into maltodextrins than did PPA, with the exception of potato starch. The four types of starches differed in the degrees to which they were converted into maltodextrins in the two reaction conditions. Maize and waxy maize starches were consistently converted into about twice as much maltodextrins than were amylomaize-7 and potato starches.

References

1. Jane J.-l.; Kasemsuwan T.; Leas S.; Zobel H. F.; Robyt J. F. *Starch/Stärke* **1994**, *46*, 121–129.
2. Kimura A.; Robyt J. F. *Carbohydr. Res.* **1995**, *277*, 87–107.
3. Sandstedt R. M.; Gates R. L. *Food Res.* **1954**, *19*, 190–199.
4. Leach H. W.; Schoch T. J. *Cereal Chem.* **1961**, *38*, 34–46.
5. Meagher M. M.; Nikolov Z. L.; Reilly P. J. *Biotechnol. Bioeng.* **1989**, *34*, 681–688.
6. Svensson B.; Larsen K.; Svendsen I.; Boel E. *Carlsberg Res. Commun.* **1983**, *48*, 529–544.
7. Stoffer B.; Frandsen T. P.; Busk P. K.; Schneider P.; Svendsen I.; Svensson B. *Biochem. J.* **1993**, *292*, 197–202.
8. Belshaw N. J.; Williamson G. *Biochim. Biophys. Acta* **1991**, *1078*, 117–120.
9. Svensson B.; Larsen K.; Gunnarsson A. *Eur. J. Biochem.* **1986**, *154*, 497–502.
10. Kim Y.-K.; Robyt J. F. *Carbohydr. Res.* **1999**, *318*, 129–134.
11. Robyt J. F.; French D. *J. Biol. Chem.* **1970**, *245*, 3917–3927.
12. Robyt J. F.; French D. *Arch. Biochem. Biophys.* **1963**, *100*, 451–467.
13. Fox J. D.; Robyt J. F. *Anal. Biochem.* **1991**, *195*, 93–96.
14. Robyt J. F.; Mukerjea R. *Carbohydr. Res.* **1994**, *251*, 187–202.
15. Mukerjea R.; Kim D; Robyt J. F. *Carbohydr. Res.* **1996**, *292*, 11–20.
16. Robyt J. F.; French D. *Arch. Biochem. Biophys.* **1970**, *138*, 662–672.