Reaction Patterns of Maize α-Amylases with Soluble Starch, Granular Starch, and Maltooligosaccharides

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ABSTRACT

Action patterns of maize α-amylases on soluble starch, granular starch, and maltooligosaccharide substrates were evaluated. Enzyme fractions with low, intermediate, and high isoelectric points (pI) had specific patterns on soluble starch. Primary reactions on starch molecules and large oligosaccharides produced large quantities of oligosaccharides with degree of polymerization (DP)7 and smaller quantities of oligosaccharides with DP8–15. Secondary reactions, identified by the point at which net consumption of DP7 began to occur, consisted of the hydrolysis of oligosaccharides with DP7 and higher to fragments of DP6 and below. Each enzyme type showed characteristic primary and secondary patterns. For high-pI and low-pI enzyme forms, secondary action patterns were equivalent to patterns obtained by hydrolysis of DP7 maltooligosaccharide. For the intermediate-pI enzymes, the reaction with DP7 was complicated by transglycosylation, resulting in the formation of higher oligosaccharides from DP4 oligomers. For all forms, reactions on starch granules differed significantly from both the primary and secondary reactions on soluble starch.

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Hydrolysis of starch by α-amylases is a complex procedure. It involves a rapid primary hydrolysis of α-1→4 bonds of amylose and amylopectin molecules and a slower secondary reaction to hydrolyze the fragments formed in the primary reaction to low molecular weight oligosaccharides (Robyt 1984). Robyt and French (1967) described the different possible types of attack on the starch molecule, as well as binding sites for *Bacillus subtilis* (Robyt and French 1963) and porcine pancreatic (Robyt and French 1970) α-amylases. Hill and MacGregor (1988) determined that, for cereal α-amylases (multichain or completely random), hydrolysis of a single bond per encounter is the predominant mode of primary attack, followed by slow, nonrandom hydrolysis of the oligomer fragments.

MacGregor and MacGregor (1985a,b) studied the action of barley α-amylase and proposed a model for starch hydrolysis by cereal α-amylases. This model contains nine binding subsites, with the catalytic site located between sites 6 and 7. By calculating the relative binding energies of the subsites, they were able to predict, quite accurately, the proportions of small oligomers formed at different stages of reaction. They postulated that this model may be applicable to the action of other cereal α-amylases as well.

We previously reported the isolation of multiple forms of α-amylase from endosperm of germinating seeds by using affinity chromatography on β-cyclodextrin epoxy-activated Sepharose 6B, followed by chromatofocusing (Warner and Knutson 1991, Warner et al 1991). The affinity-bound α-amylases, constituting approximately 50% of the total amylase activity in the endosperm, were all capable of attacking granular maize starch. Originally, four major enzyme forms (B-I through B-IV) were designated to indicate affinity binding and order of chromatofocusing elution. Elution pH values are: 5.5–5.7 (B-I); 5.1–5.4 (B-II); 4.6–4.8 (B-III); 3.9–4.1 (B-IV). Analysis of reaction products of these enzymes at the limit of their reaction on soluble starch (i.e., the point of hydrolysis at which no further change in product formation can be detected) indicated significant and reproducible differences in the action of three of the four major forms. Only B-I and B-II have equivalent final reaction patterns. Enzymes from six cultivars were isolated and fractionated (Knutson and Grove 1992), demonstrating that all four affinity-bound α-amylases are generally present in germinating maize, but relative amounts of the different forms vary widely. We also identified minor amounts of an additional enzyme form (non-B-III) with a chromatofocusing elution pH similar to that of B-III (4.4–4.6) but with a different action pattern, in all of the cultivars studied. Final action patterns of the high and low isoelectric point (pI) forms of maize α-amylases are similar and typical of what might be expected from cereal α-amylases in general, according to the nine-subsite model proposed by MacGregor and MacGregor (1985a,b). However, the pattern for the major intermediate-pI form, B-III, is quite different from typical patterns for cereal α-amylases, suggesting that the mode of attack or the number of subsites involved may differ for the different enzyme forms.

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This study was undertaken to define primary and secondary action patterns of maize α-amylases on soluble starch and to compare these patterns with known reaction patterns from other cereal α-amylases, to compare the secondary reactions products from soluble-starch hydrolysis with those obtained by action on oligosaccharide substrates, and to compare both primary and secondary soluble-starch action patterns with the action patterns on granular starch to determine whether the action patterns on soluble substrates are relevant to the action patterns of cereal α-amylases on starch granules.

MATERIALS AND METHODS

Granular maize starch, extracted by standard commercial methods and not subjected to temperatures greater than 50°C, was obtained from American Maize Products, Hammond, IN. Lintner soluble starch and maltooligosaccharides (DP1–7) were obtained from Sigma Chemical Company, St. Louis, MO. Lintner soluble starch, rather than amylose, was used as a soluble substrate so that data on reaction products would not be distorted by products emanating from chains longer than those in maize amylopectin and would, thus, be more comparable to data expected from starch granules. Additionally, soluble starch would retrograde less than amylose during slow reactions at low enzyme concentrations.

Procedures for enzyme isolation and fractionation by affinity chromatography and chromatofocusing from six maize cultivars were described previously (Warner et al 1991, Knutson and Grove 1992). Amylase activity was determined by the method of Bernfeld (1951).

Reaction conditions for the hydrolytic studies were: 100-μl aliquots of enzyme solution, containing 2–20 units (1 unit = 1 μmol of apparent maltose released per minute) of activity per milliliter, were added to 100 mg (dwb) of either soluble or granular starch or 40 mg of maltooligosaccharides in 5-ml filter-sterilized buffer (20 m*M* acetate, pH 4.5, containing 1 m*M* $CaCl_2$) to give final substrate concentrations of 20 mg/ml for starch or 8 mg/ml for oligosaccharide. No antibacterial agents were used. When the substrate was soluble starch, the solution was boiled to dissolve the starch and cooled to room temperature before adding enzyme. Otherwise, the substrates were added to buffer with no heating. Reactions were run at room temperature. Samples were kept stoppered between sampling periods to prevent evaporation. Samples containing granular starch, which rapidly settled to the bottom of the container, were shaken periodically. From this reaction mixture, 0.25-ml aliquots were taken at intervals of 0.5–672 hr for soluble starch, 1–15 days for maltooligosaccharides, or 1–27 days for granular starch. The aliquots were pipetted into 0.50 ml of absolute ethanol to terminate the enzyme action and to precipitate unreacted starch. This concentration of ethanol is sufficient to stop enzyme action and prevent microbial contamination of the hydrolyzed sample. Oligosaccharides larger than ~20 glucose residues precipitate at this concentration of ethanol. The mixture was centrifuged to settle the precipitate and filtered (Gelman Acrodisc PVDF 0.2-μm filter).

Samples were analyzed by high-performance liquid chromatography using a Dionex Carbopak PA1 carbohydrate column in a Spectra-Physics 8100 equipped with a Dionex PAD-2 pulsed amperometric detector. Oligosaccharides were separated by gradient elution: solution A was 100 m*M* NaOH (Fisher Chemical Company); solution B was 100 m*M* NaOH plus 500 m*M* NaOAC (Sigma Chemical Company). The standard gradient was 98% A at 0 time, to 70% A in 5 min, to 50% A after a total elution time of 15 min, then held at 50% A to 20 min. This gradient resolved maltooligosaccharides up to DP20 in 25 min. Calibration used standard maltooligosaccharide samples of DP1–7 as external standards. Because differences in response factors became proportionally less as the length of the oligosaccharide increased, individual response factors for oligosaccharides greater than DP7 were not calculated.

RESULTS AND DISCUSSION

Soluble-Starch Substrate

Percent of substrate hydrolyzed by the different enzyme forms, calculated from measurement of accumulation of individual soluble-reaction products, is shown in Figure 1. All reactions had minimal accumulation of oligosaccharides greater than DP15 and only slightly greater accumulation of oligosaccharides of DP10–15. This is consistent with the expected chain lengths in the substrate.

For each enzyme form, a hydrolysis limit for soluble-starch substrate was calculated. B-I and B-II enzymes had lower hydrolysis limits than those of the other enzyme forms. Those limits varied with activity level, suggesting inactivation of B-I and B-II during prolonged reaction times. For enzyme samples averaging 16 units/ml, the α-amylolysis limit for B-I and B-II was 69% of starting weight of substrate; for 10 units/ml, 46%; and for 6 units/ml, 41%. At all activity levels (2–22 units/ml), α-amylolysis limits for B-III, non-B-III and B-IV were 73–74%.

Accumulation of individual products from soluble starch was plotted for each enzyme as the mol percent of total reaction

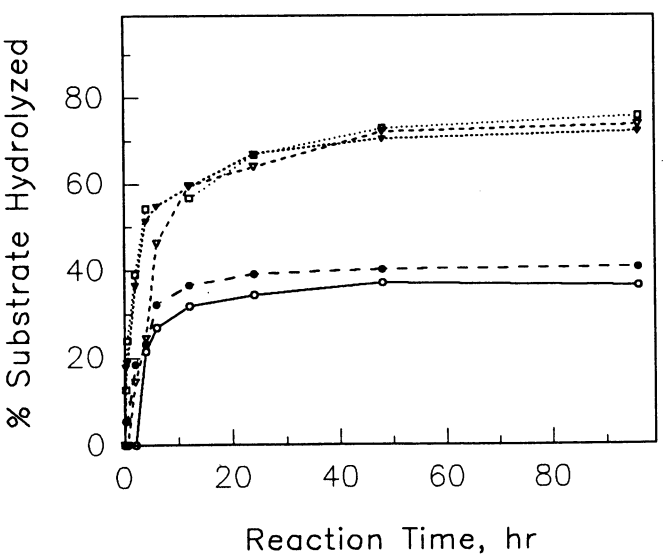

Fig. 1. Hydrolysis of soluble starch by maize α-amylases at room temperature, pH 4.5. ○ = B-I (6 units/ml); ● = B-II (7); ▽ = B-III (8); ▼ = non-B-III (9); □ = B-IV (7).

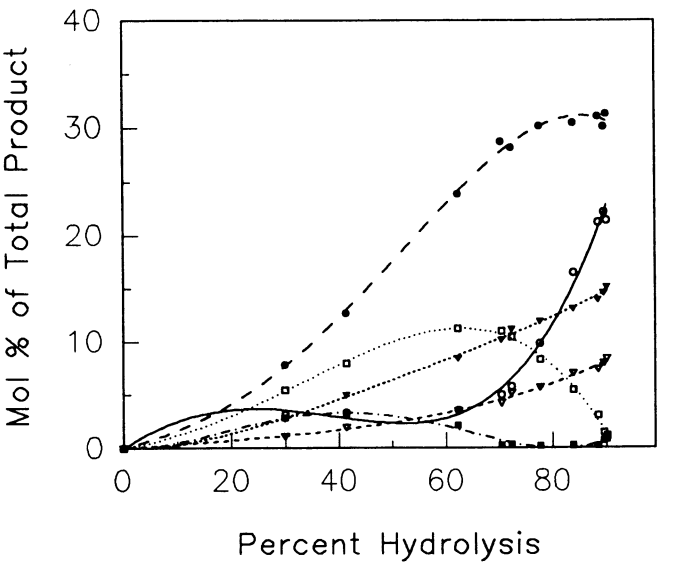

Fig. 2. Accumulation of reaction products for B-I and B-II maize α-amylases. Degree of polymerization (DP). ○ = DP1; ● = DP2; ▽ = DP5; ▼ = DP6; □ = DP7; ■ = DP8.

products versus the extent of the reaction, expressed as a percent of the α-amylolysis limit (Figs. 2–4). Such plots, as used by Torgerson et al (1979) and Kondo et al (1980), are independent of reaction time and allow data from reactions run at different levels of enzyme activity to be plotted on the same graph. Plotted values in these figures were the means of three or four samples for each enzyme.

All enzymes formed measurable amounts of oligosaccharides DP1–6 in the final product. DP7 and larger were formed in the early stages of the reactions and were subsequently hydrolyzed as reactions were allowed to continue. DP1, 2, and 5–8 showed significant changes as a proportion of total hydrolysate during the course of the reaction. DP3–4 were relatively unaffected by changes in concentration of other products. Oligosaccharides of >DP9 were observed in very small amounts and tended to reflect the behavior of DP8. Overall action patterns are summarized here, with typical reactions for each enzyme shown in the corresponding figures.

B-I and B-II (Fig. 2) show no significant differences in action patterns, which is consistent with the fact that they are not completely resolved, as shown by isoelectric focusing (Warner et al 1991). In the initial stages of the reaction, rate of accumulation of all products was linear for substrate hydrolysis. DP2 and 7 accumulated most rapidly. Net accumulation of DP7 and 8 ceased at about 70 and 60%, respectively, of the reaction limit, and net consumption of these products began. Beyond this point, rate of accumulation of DP1, 5, and 6 accelerated.

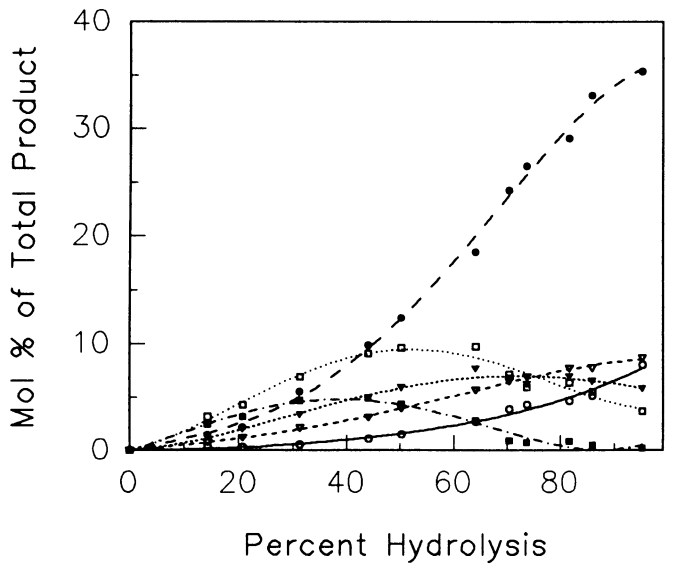

Fig. 3. Accumulation of reaction products for B-III maize α-amylases. Degree of polymerization (DP). ○ = DP1; ● = DP2; ▽ = DP5; ▼ = DP6; □ = DP7; ■ = DP8.

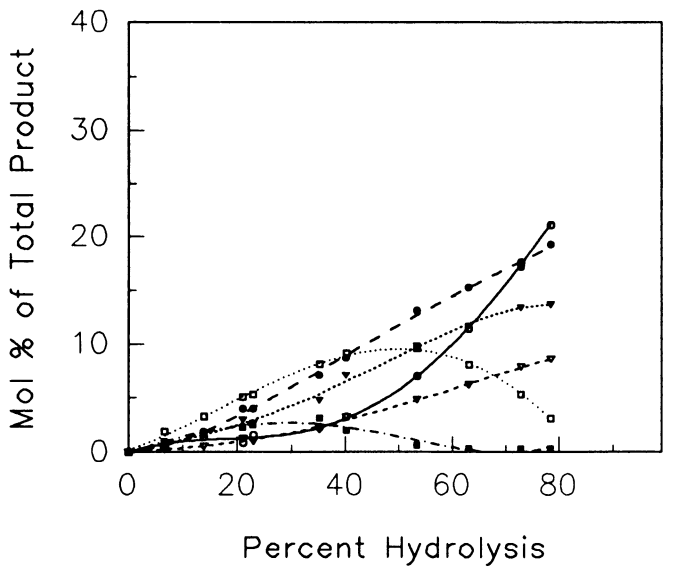

Fig. 4. Accumulation of reaction products for B-IV maize α-amylases. Degree of polymerization (DP). ○ = DP1; ● = DP2; ▽ = DP5; ▼ = DP6; □ = DP7; ■ = DP8.

B-III (Fig. 3) initially had the most rapid accumulation of DP7, which began to level off at about 40% of the reaction limit; net consumption of DP6 and 7 began at 65% of the reaction limit, after which only DP2 accumulated substantially.

Non-B-III (not shown) had an initial reaction pattern equivalent to that of B-III, but at later stages there was no significant consumption of DP6 and 7 products, and the reaction slowed substantially.

In B-IV (Fig. 4), linear accumulation of all oligosaccharides from DP1–7 occurred initially; consumption of DP7 began gradually after 50% of the reaction limit was reached. At this point, accumulation of DP1 increased.

For each enzyme, the rate of accumulation of individual oligosaccharides DP1–6 was linear up to the point at which net consumption of DP7 began. Consumption of >DP7 began before net consumption of DP7. Thus, this point was used to define the end of the primary reaction. At the highest levels of enzyme activity, this point was generally reached in 4–6 hr. For each enzyme, accumulation of oligosaccharides DP1–7 was evaluated throughout the primary reaction phase by linear regression analysis of data from at least seven reactions. Because the secondary reaction (hydrolysis of oligosaccharides of DP7 or smaller) is very slow relative to the primary reaction (MacGregor et al 1992), products resulting from the hydrolysis of oligosaccharides of DP3–7 could be ignored in these calculations. For oligosaccharides of DP2–7, the linear correlations were all 0.96% or greater. The overall mean standard error was 4.2% of the value of each component. DP1 had correlation of 0.84%, and the mean standard error was 10.8%.

Action patterns for the enzymes at this stage of reaction were compared to the barley α-amylase II action pattern reported by MacGregor and MacGregor (1985a,b) at a comparable stage of hydrolysis (i.e., 64% of substrate hydrolyzed to DP6 or smaller [Table I]). MacGregor and MacGregor (1985b) showed that the experimentally determined action pattern for barley α-amylase II agrees well with their predicted pattern for a nine-subsite model calculated from binding energies at the enzyme subsites. Data in Table I shows that the maize enzymes had characteristic patterns that differed from the barley α-amylase II pattern as well as from each other. Compared to the barley enzyme, patterns for the high-pI forms (B-I and B-II) produced more oligosaccharides of DP2 content and less of DP6 and 7. Intermediate-pI (B-III and non-B-III) forms produced more oligosaccharides of DP4 and less of DP6. The low-pI form (B-IV) produced more oligosaccharides of DP1 and less of DP6.

Primary action patterns of all of the enzymes on soluble starch were consistent with the nine-subsite model of MacGregor and MacGregor (1985a,b). Although product mixtures did not correspond exactly with those reported by MacGregor and MacGregor for barley starch, the differences observed are likely due

TABLE I
Oligosaccharides (DP1–7) Produced During Primary Reaction[a]

Fraction	DP1	DP2	DP3	DP4	DP5	DP6	DP7	>DP7
B-I	6	38	10	4	5	13	17	6
B-II	7	40	10	4	6	16	14	3
B-III	4	23	10	11	7	12	19	14
Non-B-III	3	24	10	10	8	13	20	14
B-IV	11	23	8	6	8	17	20	7
Barley α-amylase II[b]	5	29	9	7	6	22	23	

[a] Mol percent of total product.
[b] MacGregor and MacGregor (1985a,b). Original values recalculated to mol percent. Values reported for >6 DP were calculated as DP7.

to differences in binding energies of individual subsites. Differences in binding energies obviously exist between different forms of maize α-amylase as well, inasmuch as the pattern for the high-pI (B-I, B-II) and low-pI (B-IV) forms of maize α-amylase differed from each other more than either differed from barley α-amylase II. Furthermore, the pattern for the intermediate-pI (B-III) enzyme varied substantially from those of the other maize α-amylases.

The final product mixtures at the α-amylolysis limit are shown in Table II. Again, three characteristic patterns were found. The significant differences of the three enzyme forms at this point can be most readily observed from the relative proportions of DP1 and 2. Formation of DP1 by B-III and non-B-III enzymes remained very low throughout the hydrolysis.

Individual product totals from primary reactions were subtracted from the final reaction totals to calculate product composition of the secondary reaction phase. These values are shown in Table III. The secondary action patterns for high-pI and low-pI enzymes were similar; DP1 was the predominant product formed. In contrast, DP1 formation was very low for intermediate-pI enzymes; DP2 was the predominant product.

The low DP1 content found in reaction mixtures from B-III and non-B-III suggests the possibility that these intermediate-pI enzymes have their catalytic sites between sites 7 and 8, rather than between sites 6 and 7 (assuming a nine-subsite model). Such a configuration would preclude production of DP1 whenever all subsites are occupied; this predominantly occurs during the primary reaction. The likelihood of such a configuration was evaluated by calculating the composition of the hypothetical product mixture that would result from random binding of a DP20 substrate to an enzyme with the catalytic site between 7 and 8 for all possible encounters in which all nine subsites are occupied. The calculated values for this mixture were 24, 15, 6, 9, 3, 26, and 16 mol% for DP2–8 oligosaccharides, respectively. This corresponds closely to values in Table I for the primary action patterns of B-III and non-B-III enzymes.

Oligosaccharide Substrates

Products of reaction of individual enzymes with maltooligosaccharides of DP4–7 are shown in Table IV. Reactions are summarized here.

Only B-III reacted to a significant degree with DP4 or 5. A measurable accumulation of DP5 and 6 was found in the reaction mixture from DP4, indicating the occurrence of transglycosylation with this enzyme. This is consistent with the finding of MacGregor et al (1992): where transglycosylation occurs, it is more evident with smaller oligosaccharides that are hydrolyzed with more difficulty.

B-I and B-II reacted moderately on DP6. B-III reacted extensively on DP6. B-IV showed no action on DP6 in 14 days.

TABLE II
Oligosaccharides (DP1–7) Present After Final Reaction[a]

Fraction	DP1	DP2	DP3	DP4	DP5	DP6	DP7	>DP7
B-I	18	38	9	4	8	16	6	1
B-II	18	38	9	4	8	16	6	2
B-III	7	44	17	15	7	5	3	1
Non-B-III	6	32	14	14	9	10	11	3
B-IV	28	24	9	8	10	16	4	1

[a] Mol percent of total product.

TABLE III
Oligosaccharides (DP1–7) Produced by Secondary Reaction[a]

Fraction	DP1	DP2	DP3	DP4	DP5	DP6	DP7	>DP7
B-I	43	24	9	2	10	17	...	...
B-II	40	28	5	2	11	13	...	...
B-III	9	52	19	15	5	...	...	...
Non-B-III	10	41	19	17	10	...	...	...
B-IV	42	19	8	8	11	12	...	...

[a] Mol percent of total product.

B-I, B-II, and B-III converted essentially all of DP7 to smaller oligomers. B-IV reacted very slowly. The slow reaction of B-IV with DP7 was unexpected because this fraction readily hydrolyzed DP7 in the secondary phase of its action on soluble starch.

Non-B-III did not react significantly with any of the oligosaccharides in 14 days.

Overall, reactions of high (B-I, B-II) and low (B-IV) pI enzymes with DP7 oligosaccharides gave results that correlated well with those for the secondary reactions of the same enzymes on soluble starch. There was less agreement for the intermediate-pI enzymes (B-III, non-B-III). This difference may reflect the apparently higher level of transglycosylation observed with intermediate-pI enzymes.

Granular Starch Substrate

Rate of reaction of B-I, B-III, and B-IV on granular maize starch is shown in Figure 5. Because the rates of reaction on granular starch were slower than those on soluble starch, the time of reaction was plotted as days rather than hours. B-IV was substantially more efficient at granule hydrolysis than were the other enzymes. B-IV hydrolyzed equivalent or greater amounts of starch than the other enzymes with only one-third the activity. This difference suggests greater accessibility of starch molecules at the granule surface to the active sites of B-IV, possibly as a result of more effective adsorption of the enzyme to the granule surface. Action patterns, which remained essentially constant throughout the course of the reaction, were evaluated by linear regression and compared to the pattern reported by MacGregor

TABLE IV
Action of Maize α-Amylase on Maltooligosaccharides[a]

Substrate/ Enzyme Fraction	Substrate Consumed, %	DP1	DP2	DP3	DP4	DP5	DP6
DP4 B-III	28	8	87	...	...	1	4
DP5 B-III	33	3	60	34	4	...	...
DP6 B-I,II	59	15	54	15	17	...	...
DP6 B-III	97	3	59	16	22	...	...
DP6 Non-B-III	21	1	53	16	29	...	...
DP7 B-I,II	92	40	24	5	5	12	13
DP7 B-III	83	4	34	21	10	7	24
DP7 Non-B-III	11	4	43	20	17	15	...
DP7 B-IV	29	48	19	6	7	18	1

[a] Mol percent of total product.

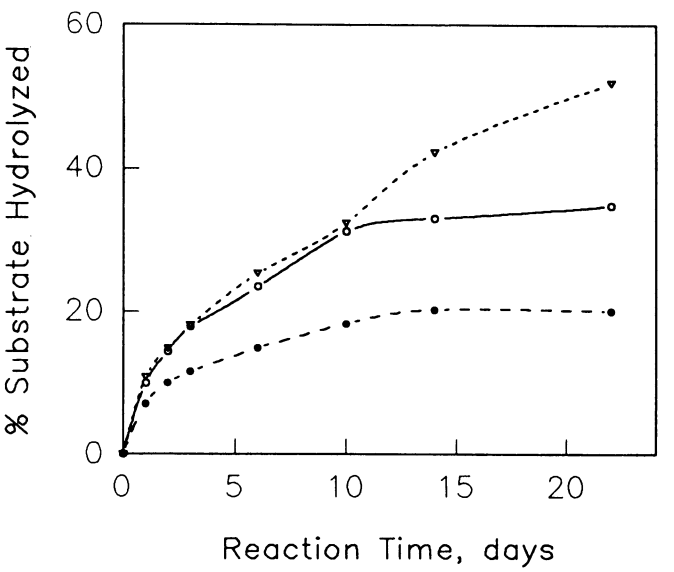

Fig. 5. Hydrolysis of granular starch by maize α-amylases at room temperature, pH 4.5. ○ = B-I (17 units/ml); ● = B-III (18); ▽ = B-IV (6).

TABLE V
Action of Maize α-Amylases (DP1-7) on Granular Starch[a]

Fraction	DP1	DP2	DP3	DP4	DP5	DP6	DP7
B-I	26	49	8	8	4	4	1
B-III	9	61	19	7	2	1	...
B-IV	27	39	11	12	5	6	1
Barley α-amylase II[b]	14	44	12	15	5	4	5

[a] Mol percent of total product.
[b] MacGregor and MacGregor (1985a). Enzyme acting on small barley starch granules.

and MacGregor (1985a) for barley α-amylase II enzyme on small starch granules at 21% starch solubilization (Table V). As with soluble starch, a different action pattern was observed for each enzyme, but patterns with granular starch were not equivalent to those with soluble starch. Hydrolysis of granular starch produced higher proportions of DP2 and lower proportions of DP6 than were found with soluble starch. B-I predominantly produced DP1 and 2 in an approximate ratio of 1:2. B-III was the only enzyme for which the action pattern on granular starch was similar to that on soluble starch: very little DP1 and a high proportion of DP2. The B-IV action pattern was most similar to that of the barley enzyme, with a higher proportion of DP4 than the other maize enzymes.

Significantly, there was essentially no accumulation of DP7 at any stage of granular starch hydrolysis by any of the maize enzyme forms. It appears that all of these enzymes hydrolyzed DP7 more rapidly than they did granular starch.

CONCLUSIONS

High-, intermediate-, and low-pI forms of maize α-amylase each have characteristic action patterns on soluble starch, oligosaccharides, and starch granules. These patterns are valuable for characterization and identification of individual enzyme forms. Patterns for high-pI and low-pI forms (B-I, B-II, B-IV) are consistent with the nine-subsite model of MacGregor and MacGregor (1985a,b) for cereal α-amylases. Patterns for intermediate-pI forms (B-III, non-B-III) also indicate a nine-subsite model but suggest a different location for the catalytic site, possibly between subsites 7 and 8.

For each enzyme form, differences between action patterns on soluble starch and on granular starch are substantial. As a result, determinations of the action patterns on soluble substrate are of limited use for predicting relative rates of hydrolysis or reaction products for starch granules. This point has been demonstrated, with regard to reaction rates only, by Sun and Henson (1991) for the different types of starch-degrading enzymes found in germinating seeds (α-amylase, β-amylase, α-glucosidase, and debranching enzymes).

The significant differences in reaction products and variation in rates of granule hydrolysis relative to soluble-starch activity for the different forms of maize α-amylases suggests varying roles in the germination process, as well as multiple roles for individual forms at different stages of germination.

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