A Comparison of the Catalysis of Starch Components by Isoenzymes from the Two Major Groups of Germinated Wheat α-Amylase[1]

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ABSTRACT

α-Amylase isoenzymes from the two major groups (GI and GIII) in germinated wheat were separated and purified by a two-step process consisting of affinity chromatography and chromatofocusing. The two groups, at equivalent enzymic activities, were compared for their ability to hydrolyze a variety of starch substrates. No significant differences were found in the catalytic hydrolysis of β-limit dextrin, amylopectin, or amylose, as indicated by product distributions evaluated by high performance aqueous gel permeation chromatography. At 37°C, only the GIII group adsorbed onto large and small granules. Both groups solubilized starch; small granules were degraded faster than large granules. At 2°C, the adsorption characteristics onto starch granules were identical for both groups. The GI and GIII isoenzymes had identical effects in lowering the amylograph viscosity of a wheat flour and in increasing its gassing power, which suggests that the action pattern of the two groups must be very similar.

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Germinated wheat contains two main groups of α-amylase isoenzymes (Olered and Jönsson 1970, Kruger 1972). The group in largest proportion has pI values between 6.0 and 6.5 and is sometimes referred to as the major, basic pI, or "germinated" α-amylase. The second group represents a much smaller proportion of the total α-amylase activity and is referred to as the minor, acidic pI, or "green" α-amylase. This group has pI values between 4.5 and 5.1, and isoenzymes with identical isoelectric points are also present in developing wheat kernels (Tkachuk and Kruger 1974; Marchylo et al 1976, 1980b; Sargeant 1979). Each group is heterogeneous, with the number of isoenzymes resolved depending on the separatory technique used. To date, optimum resolution of α-amylase isoenzymes has been achieved with polyacrylamide gel isoelectric focusing (PAG-IEF). By this technique, germinated wheat α-amylase has been separated into two main and one minor group of components, which have been referred to as GIII (germinated), GI (green), and GI (pI intermediate to the two main groups) groups, respectively (Marchylo et al 1980a, b; Marchylo and Kruger 1983).

The physiological reasons for the existence of two main groups of α-amylases are still uncertain. Sargeant and Walker (1978), however, found that the more basic pI isoenzymes adsorb onto undamaged starch granules and subsequently degrade them, whereas the acidic pI isoenzymes are not adsorbed and did not

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degrade starch granules. Moreover, the acidic pI isoenzymes from developing wheat kernels behave similarly to the germinated wheat counterparts. Sargeant and Walker postulated that the main function of the germinated α-amylases may be to initiate degradation of the starch granule, whereas the role of the green amylases might be to assist in degrading soluble oligosaccharides released from the starch granule. On the other hand, Weselake and Hill (1983) found that the green α-amylases are much more effective than the germinated α-amylases in adsorbing onto and solubilizing starch granules. The α-amylase system in barley behaves similarly (MacGregor and Ballance 1980a); i.e., the acidic pI α-amylase system (minor group) is much more efficient than the more basic pI α-amylase system (major group) in hydrolyzing large starch granules.

Aside from considerations regarding separate physiological roles, little is known concerning the functional roles of the two main α-amylase groups in breadmaking. For example, green α-amylases are less heat-stable than are germinated α-amylases (Marchylo et al 1976) and therefore should be inactivated earlier, as the temperature rises during the oven stage of breadmaking. As a consequence, green α-amylases should not degrade the starch components of a dough to the same extent as the germinated α-amylase. On the other hand, in a long fermentation breadmaking process, the role of the green α-amylases could be quite important.

The goal of this study was to obtain more information on the physiological and potential functional roles in breadmaking of wheat α-amylase. The effects of purified α-amylase isoenzymes from the two main groups in degrading various carbohydrate substrates were examined. Instead of the conventional method of purifying α-amylase (heat-treatment, acetone-fractionation, glycogen-complex formation, and ion-exchange chromatography [Kruger and Tkachuk 1969]), two new techniques, affinity chromatography and chromatofocusing, were used for rapid purification of α-amylase isoenzymes.

MATERIALS AND METHODS

Germination of Wheat

Canadian hard red spring wheat (cultivar Neepawa) was sterilized with 1% sodium hypochlorite, rinsed thoroughly, steeped for 2 hr, and germinated for 120 hr at 18° C in a humidity cabinet. The sample was then air-dried and ground in a Udy cyclone grinder (Udy Corp., Boulder, CO).

α-Amylase Determination

α-Amylase activity was determined with a Perkins-Elmer model 191 grain amylase analyzer and β-limit dextrin substrate, as described by Kruger and Tipples (1981).

Purification of Wheat α-Amylase Isoenzymes

α-Amylase isoenzymes were purified from extracts of germinated wheat by a two-step process, consisting of affinity chromatography on cycloheptaamylose-epoxy-sepharose-6B (Silvanovitch and Hill 1976) followed by chromatofocusing (Marchylo and Kruger 1983). Details of the purification follow.

Extraction of enzyme. To 45 g of ground germinated wheat was added 150 ml of 0.02*M* sodium acetate, pH 5.2, containing $10^{-3}M$ $CaCl_2$. The mixture was stirred for 1 hr and then centrifuged at 15,000 rpm for 15 min at 5° C; this centrifugation step was repeated on the supernatant and the extract was stored at 5° C. The final volume of 100–115 ml was filtered through Whatman no. 1 filter paper before affinity chromatography.

Affinity chromatography. This was done according to the procedure of Silvanovitch and Hill (1976), using cycloheptaamylose-epoxy-sepharose 6B with slight variations. Cycloheptaamylose-epoxy-sepharose 6B was packed into a 0.8 × 30-cm column and equilibrated with 0.02*M* sodium acetate, pH 5.2, containing $10^{-3}M$ $CaCl_2$, at a flow rate of 17 ml/hr. The sample was applied, and the starting buffer was passed through the column overnight (approximately 16 hr), to remove as much contaminating protein as possible. The column was washed further for 1.5 hr with 0.02*M* sodium acetate buffer, pH 5.2, containing 0.3*M* sodium chloride, followed by equilibrating buffer for 1 hr. The α-amylase was removed from the column with 125 ml of cycloheptaamylose, at a concentration of 8 mg/ml. Eluent fractions (7.5 ml) were collected during each stage with a fraction collector. The column was then reequilibrated with starting buffer for the next run.

Chromatofocusing. The α-amylase affinity peak was concentrated with an Amicon ultrafiltration apparatus (Amicon Corp., Lexington, MA) to 3 ml and was subjected to chromatofocusing, as described by Marchylo and Kruger (1983). Bovine serum albumin (1 mg/ml) was included in the starting buffer, as this conferred stability to the resolved α-amylase components.

Amylograph Viscosity

Sixty-five grams of flour (14% moisture basis) and 450 ml of distilled water were used with the Brabender amylograph and the pin stirrer (AACC 1962). Peak viscosity is reported in Brabender Units. Enzyme extracts of varying concentration were added to the distilled water before the amylograph determination was performed.

Gassing Power

Gassing power was determined on a 10-g flour sample by the AACC Pressuremeter Method, using a modified pressuremeter (Transactions, AACC 1951). Enzyme extracts were diluted with distilled water so that the final volume was 7 ml, to which was added 0.35 g of yeast. The yeast mixture and 10 g of flour were mixed and placed in the pressuremeter. Results are reported as millimeters pressure for total gas evolved after 6-hr fermentation.

Preparation and Fractionation of Starch Granules

Starch granules were prepared according to the procedure of MacGregor (1979), using ground Neepawa wheat. This consisted of a preliminary fractionation to obtain a crude starch preparation, followed by fractionation into large and small granules using an elutriation column. $HgCl_2$ was not included in the initial steep water, to avoid possible inhibitory effects on α-amylase components. Purified starch granules were washed several times with ethyl alcohol and then with diethyl ether, rather than solvent removal by freeze-drying. Microscopic examination indicated that the small starch granules were completely free of large starch granules. The large granule preparation contained 6% small starch granules. All granules were birefringent.

Adsorption of α-Amylase

Twenty-five milligrams of starch was placed in a capped centrifuge tube, and 10 ml of enzyme in 0.05*M* sodium acetate, pH 5.5, containing $10^{-3}M$ $CaCl_2$, was added. The solution also contained bovine serum albumin, 0.5 mg/ml, because this concentration maintains the stability of α-amylases (MacGregor and Ballance 1980b). The suspension was gently mixed with a Lab-Line rotary shaker (Labindustries, Berkeley, CA) in a water bath at 37 or 4° C. The suspension then was centrifuged for 10 min at 5,000 rpm. Part of the supernatant was assayed for α-amylase and compared with the activity of the original extract that contained no starch. Another portion of the supernatant was analyzed for total solubilized carbohydrates by an automated orcinol-sulfuric acid procedure (LaBerge et al 1973).

High Performance Aqueous Gel Permeation Chromatography

The procedure and equipment for the analysis of the molecular weight distribution of hydrolytic products resulting from the breakdown of soluble starch components were identical to that described previously (Kruger and Marchylo 1982). A 60 × 0.75 cm Spherogel-TSK 3000 SW column (Altex Scientific, Berkeley, CA) was calibrated with the following dextrins obtained from Pharmacia (Canada), Ltd., Dorval, Quebec: T-110 (mol wt 1.1×10^5), T-70 (mol wt 7.1×10^4), T-40 (mol wt 4.1×10^4), and T-10 (mol wt 9.9×10^3). Void volume was determined with thyroglobulin (6.7×10^5) and β-limit dextrin. The substrates chosen for analyses were β-limit dextrin, prepared as described previously (Kruger and

Tipples 1981); amylopectin, A-grade (Calbiochem, La Jolla, CA); and amylose, type III, from potato (Sigma Chem. Co., St. Louis, MO).

RESULTS

Purification of Wheat α-Amylase Isoenzymes

The first step in the purification of wheat α-amylase was affinity chromatography. A typical profile for the purification of wheat α-amylase is shown in Figure 1. The recovery of α-amylase was 66% of that applied to the column. With increasing use of the column, this percentage decreased to about 45%. Between 2.2 and 3.3% of the α-amylase activity was found in the through peak, which indicates that at least one-third of the activity is lost, perhaps by irreversible adsorption to the column. The specific activity increased 855-fold by this purification step. Weselake and Hill (1983) found that β-amylase is not present in the α-amylase peak removed by addition of β-cyclodextrin. In our study, the affinity peak was tested for presence of β-amylase by first subjecting an aliquot to PAG-IEF (pH 3.5–9.5). α-Amylase and β-amylase isoenzymes then were detected by a a β-limit dextrin-plate (α-amylase) or starch-plate (α- and β-amylase) technique (Marchylo 1980b). No additional bands were found when the β-limit dextrin zymogram was compared with the starch zymogram, indicating the absence of β-amylase components. The molecular weight distribution of products formed by α-amylase breakdown of β-limit dextrin also confirmed the absence of β-amylase.

The second stage of the wheat α-amylase purification was chromatofocusing. A typical chromatofocusing run is shown in Figure 2. Five main peaks with apparent pI values of 6.22, 5.94, 5.38, 5.26, and 4.95 were found. PAG-IEF analysis (pH 3.5–9.5) of peak fractions confirmed that the peaks with apparent pI values of 6.22 and 5.94 were GIII components, whereas the peaks with apparent pI values of 5.38, 5.26, and 4.95 were GI components. Fractions from within peak 2 (apparent pI 5.94) and peak 4 (apparent pI 5.26) were selected to represent the GIII and GI groups, respectively. This ensured no cross-contamination between groups (Fig. 3).

Effect of GI and GIII α-Amylases on Starch and Starch Components

The abilities of the GIII α-amylases (apparent pI 5.94) and GI α-amylases (apparent pI 5.26) to catalyze the hydrolysis of starch or soluble starch components were compared, using several different methods. Changes in molecular weight resulting from the breakdown of amylopectin, β-limit dextrin, and amylose were studied, using high performance aqueous gel permeation chromatography. The adsorption behavior of the two components onto large and small wheat starch granules was ascertained. Effects on the amylograph and gassing power properties of a wheat flour were also determined for increasing amounts of the two groups.

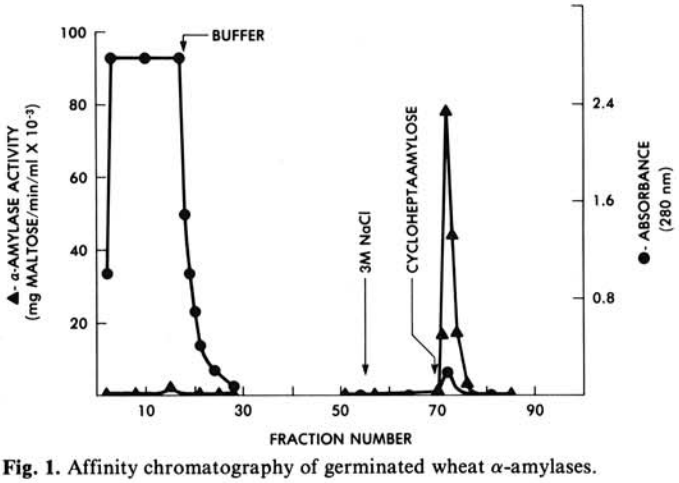

Fig. 1. Affinity chromatography of germinated wheat α-amylases.

β-Limit Dextrin, Amylopectin, and Amylose Hydrolysis

Enzyme dilutions were chosen so that equal enzyme activities of each group were compared. Changes in molecular weight due to hydrolytic breakdown of 0.5% β-limit dextrin, 0.5% amylopectin, and amylose due to α-amylase groups GI and GIII are shown in Figure 4, and the change in percentage distribution of selected molecular weight products from hydrolysis of these substrates is shown in Figure 5. No major differences were observed in the manner by which the α-amylase groups degraded β-limit dextrin

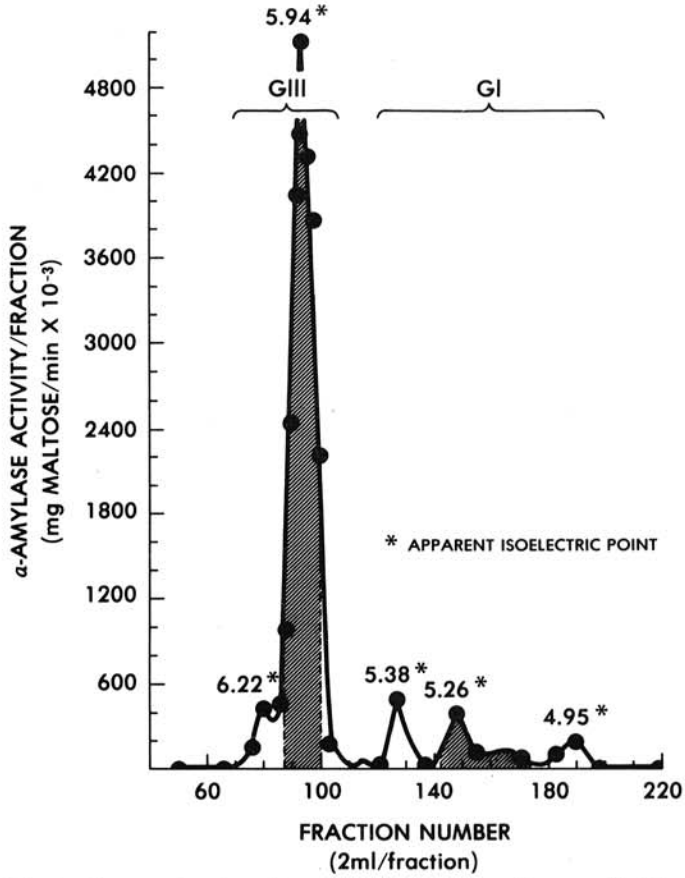

Fig. 2. Chromatofocusing of germinated wheat α-amylases purified by affinity chromatography. The hatched area indicates the fractions chosen to represent GI and GIII groups.

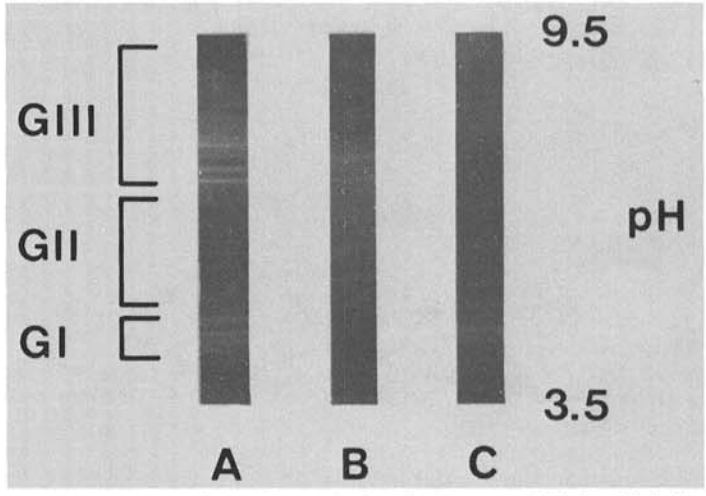

Fig. 3. Polyacrylamide gel isoelectric focusing between pH 3.5 and 9.5 of α-amylases in: A) a five-day germinated Neepawa wheat extract, B and C) peak 2 (apparent pI 5.94) and peak 4 (apparent pI 5.26), respectively, resolved by chromatofocusing.

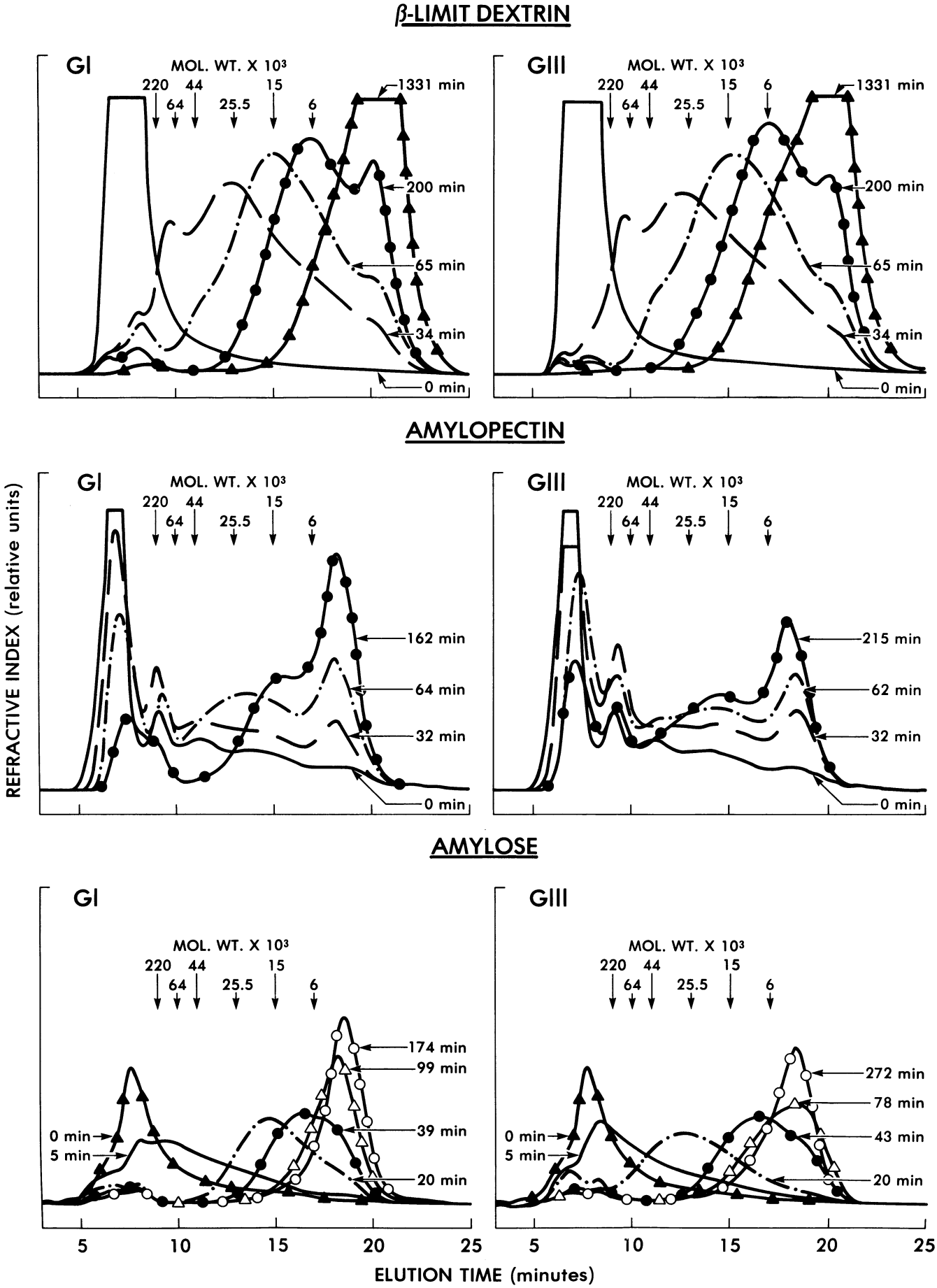

Fig. 4. Molecular weight distribution of products formed at increasing reaction times due to GI and GIII germinated wheat α-amylase hydrolysis of β-limit dextrin, amylopectin, and amylose. Void volume for the column was 7.2 ml (elution, 7.2 min).

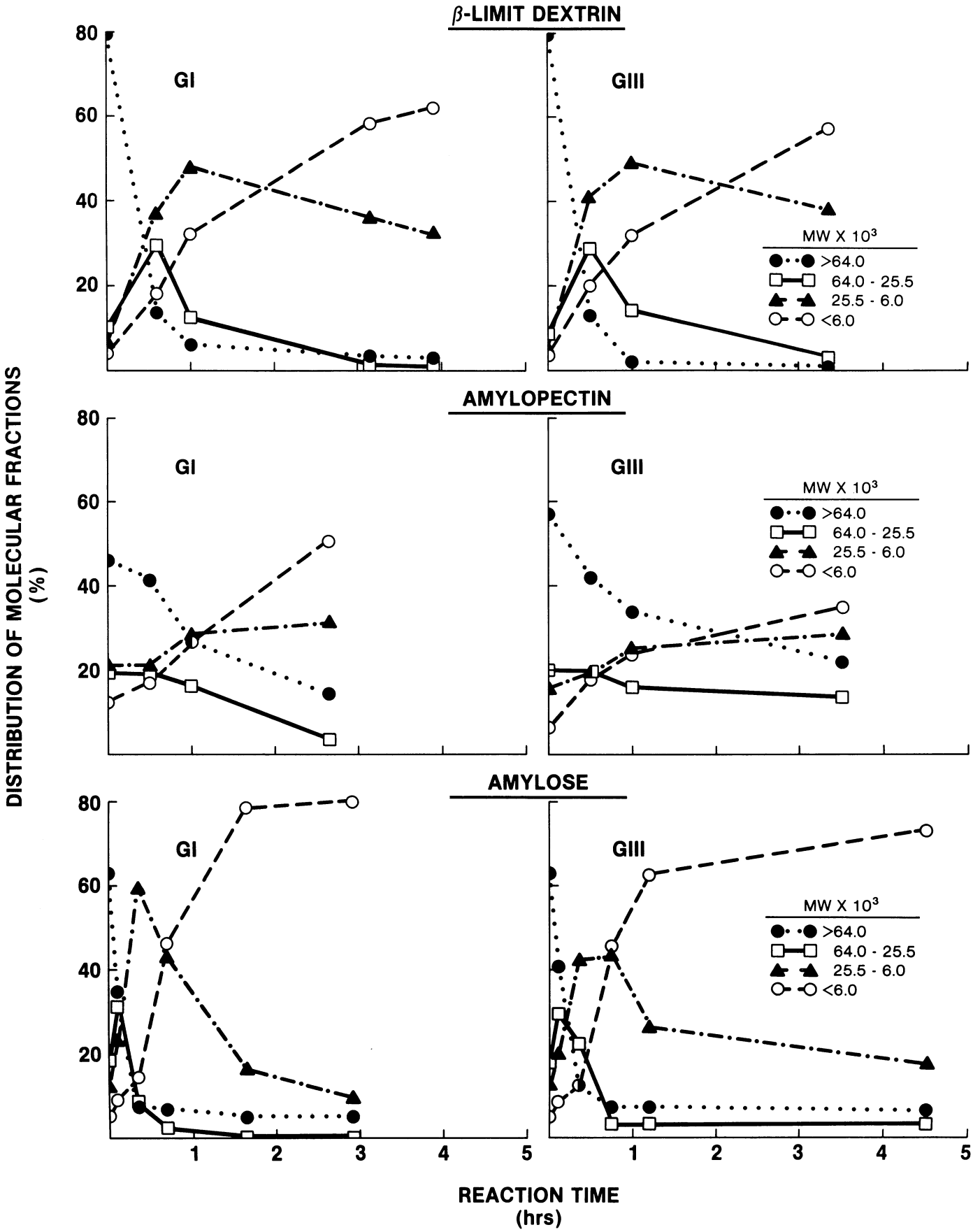

Fig. 5. Percentage of distribution of products in different molecular weight fractions from GI and GIII wheat α-amylase hydrolysis of β-limit dextrin, amylopectin, and amylose.

(Figs. 4 and 5). The only discernible difference was the ability to the GIII group to more effectively break down a core of high molecular weight dextrins that appeared to be resistant to α-amylase attack. This is indicated by a greater amount of residual carbohydrate in the through peak of the chromatograms resulting from GIII hydrolysis at the later reaction times. The catalytic breakdown pattern with reaction time for both α-amylase groups was very similar to that found recently for a purified preparation of α-amylase that contains all of the isoenzymes (Kruger and Marchylo 1982). Initially, the high molecular weight material above the exclusion limit of the column broke down rapidly. This phase of the reaction is not evident in Figure 4. The successive stages of the reaction were characterized by the gradual formation of intermediate molecular weight products, which decreased in size concomitant with the formation of the very low molecular weight sugars and simple dextrins.

Catalysis of potato amylopectin is shown in Figs. 4 and 5. Unlike β-limit dextrin, this substrate initially had a molecular weight distribution that varied widely in size. More than 30% of the amylopectin had molecular weights lower than the upper exclusion limits of the column. This made it more difficult to follow the progression of the hydrolytic breakdown from high to low molecular weights. In general, however, the two groups, GI and GIII, behaved similarly in the breakdown of this substrate. As is more evident in Figure 5, the rate of reaction of the GIII group appeared to slow slightly with time, compared to that of the GI group. This may be due to slight inactiviation of the enzyme with time rather than an actual product inhibition. In contrast to the breakdown of β-limit dextrin, the GI and GIII groups degraded amylopectin to intermediate and low molecular weight dextrins of less than 6×10^{-3} while still maintaining a substantial proportion (14–23%) of amylopectin with a molecular weight greater than the exclusion limits of the column.

Only a small proportion of amylose substrate was soluble in buffer. Equivalent concentrations, however, were used to compare effects of the two α-amylase groups. The amylose that dissolved (Fig. 4) contained a symmetrical distribution of macromolecules, which were largely excluded from the aqueous size exclusion column. Degradation by both α-amylase groups was very similar (Figs. 4 and 5). The changing molecular weight distribution of products was different, however, from that of β-limit dextrin and amylopectin hydrolysis. Whereas these latter substrates formed intermediate molecular products simultaneously with production of low molecular products, amylose hydrolysis was characterized by the progressive degradation of a more uniform and symmetrical product that decreased continually in molecular weight. This suggests that initial scission of glycosidic bonds in the interior of the initial substrate and its earlier degradation products was favored over cleavage near the ends of substrate chains.

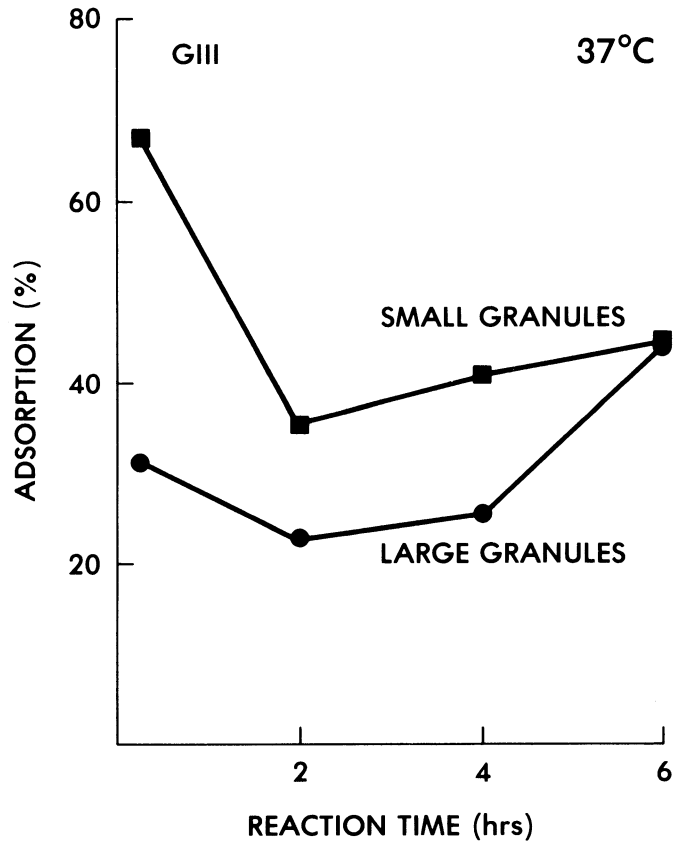

Fig. 6. Percentage of adsorption at 37° C of GIII α-amylase from germinated wheat onto small and large starch granules.

Starch Granule Hydrolysis

The adsorption and solubilization of large and small wheat starch granules by equivalent activity levels of GI and GIII wheat α-amylases were examined at 37° C. The adsorption characteristics of the GIII α-amylase are shown in Figure 6. Adsorption was initially twice as great onto small granules, but by 6 hr, the adsorption was approximately equal for both types of granules.

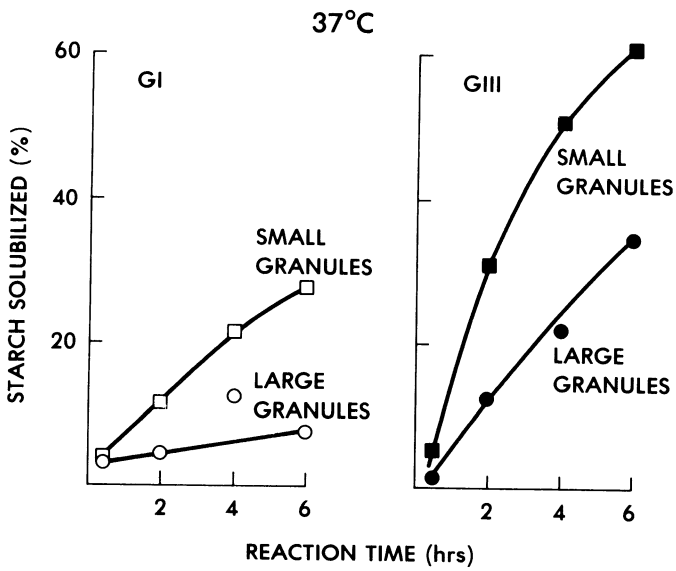

Fig. 7. Percentage of starch solubilized at 37° C by GI and GIII germinated wheat α-amylase hydrolysis of large and small starch granules.

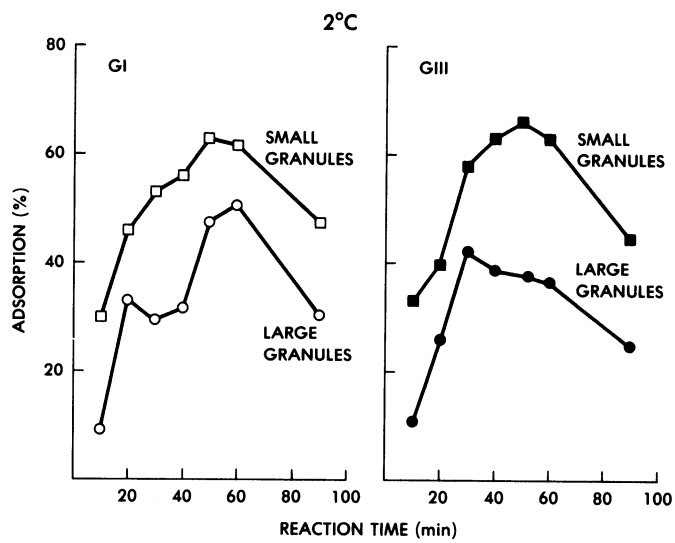

Fig. 8. Percentage of adsorption at 2° C of GI and GIII α-amylases from germinated wheat onto small and large starch granules.

After overnight incubation (not shown), adsorption onto large granules became greater. This is probably not a true change in adsorption characteristics but reflects the increased degradation and decrease in the number of small starch granules. In contrast to the substantial absorption of the GIII α-amylase, there was no adsorption by the GI α-amylase onto either the large or small starch granules at 37° C. This did not seem to be a prerequisite for starch granule degradation, however, as shown in Figure 7. Thus, the GI and GIII α-amylases hydrolyzed both types of granules. Small granules were preferentially hydrolyzed in both cases. GIII α-amylase was more effective than GI α-amylase in degrading starch granules. Thus, after 6 hr of reaction, 61% of the small starch granules were solubilized by the GIII component, compared to 27.9% by the GI component.

To evaluate the effect of temperature, adsorption experiments were done at 2° C also. The most noticeable difference was that the GI α-amylase was strongly adsorbed (Fig. 8) to approximately the same level as the GIII α-amylase. In addition, adsorption increased to a maximum with reaction time, whereas at 37° C, adsorption decreased to a minimum with reaction time and then increased.

Amylograph Peak Viscosity Changes

Increasing amounts of purified GI and GIII α-amylase were added to a hard red spring wheat flour, and the change in amylograph peak viscosity was observed. Initial viscosity of the wheat flour was 790 BU. As shown in Figure 9, at equivalent concentrations of enzymic activity as determined by the nephelometric method, both groups had identical effects in decreasing amylograph peak viscosity. The observed decrease was curvilinear with increasing concentration of enzyme.

Gassing Power Changes

The effect of increased amounts of the individual α-amylase isoenzyme groups on the gassing power of a hard red spring wheat flour is shown in Figure 10. At equivalent enzyme activities, the effects of both components are almost identical.

DISCUSSION

Multiple forms of α-amylase, representative of the two major isoenzyme groups in germinated wheat, were isolated by a convenient procedure consisting of affinity column followed by chromatofocusing. The advantage of both of these procedures is that the isolated fractions are present in small volumes of eluent, in contrast to conventional isolation procedures, such as ion-exchange chromatography. PAG-IEF analysis of peak 2 with apparent pI 5.94 showed that this peak consisted primarily of three GIII α-amylase isoenzymes and that the fourth peak, which eluted at a pH of 5.26, consisted primarily of two GI α-amylase isoenzymes (Fig. 3). Overlap between the two groups was negligible, which agrees with previous studies (Marchylo and Kruger 1983).

The effects of the GI and GIII groups on starch substrates were extremely similar. No significant differences could be found in degradation of β-limit dextrin, amylopectin, or amylose. This finding would seem to rule out conjecture that the formation of two groups is required for separate functional roles in either degrading intact starch granules or breaking down oligosaccharides once they have become solubilized. Differences were found in adsorption characteristics of the GI and GII α-amylases, which might suggest that only the GIII component can degrade starch granules, as suggested by Sargeant and Walker (1978). In our study, however, adsorption was not a prerequisite for degradation of starch granules by the GI component. Furthermore, adsorption characteristics highly depended on temperature. Although no binding was evident at 37° C, more than 60% GI adsorption was found at 2° C. Small granules were preferentially hydrolyzed over large granules at 37° C. A similar finding was reported by MacGregor and Ballance (1980a) for degradation of barley starch granules by malted barley α-amylase. They also found that the green (minor) α-amylase component of barley is more effective than the germinated (major) component with respect to this degradation. Similar results were found by Weselake and Hill (1983) with a wheat system. Our results support the findings of Sargeant and Walker that the germinated GIII wheat α-amylase

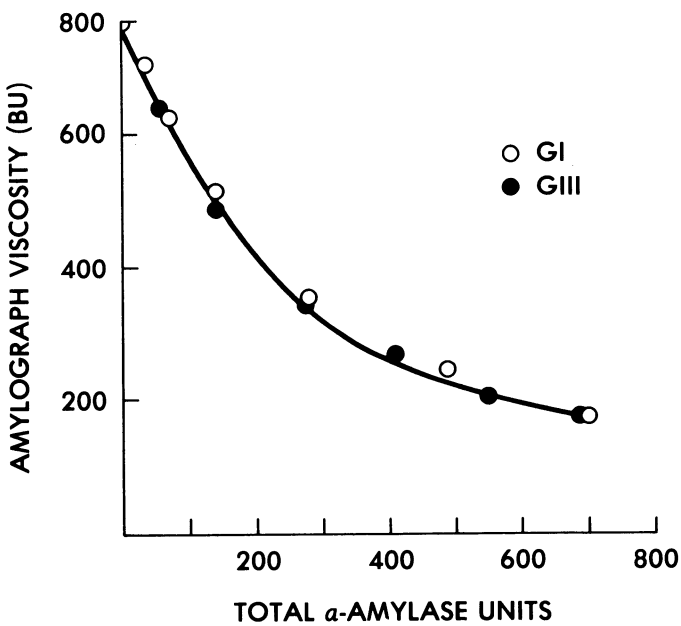

Fig. 9. Decrease in the amylograph viscosity of wheat flour with increasing addition of GI and GIII α-amylases from germinated wheat. One unit of α-amylase denotes the amount that hydrolyzes 1 mg of maltose/min × 10^{-3} at 37° C from reduced starch (Kruger and Tipples 1981).

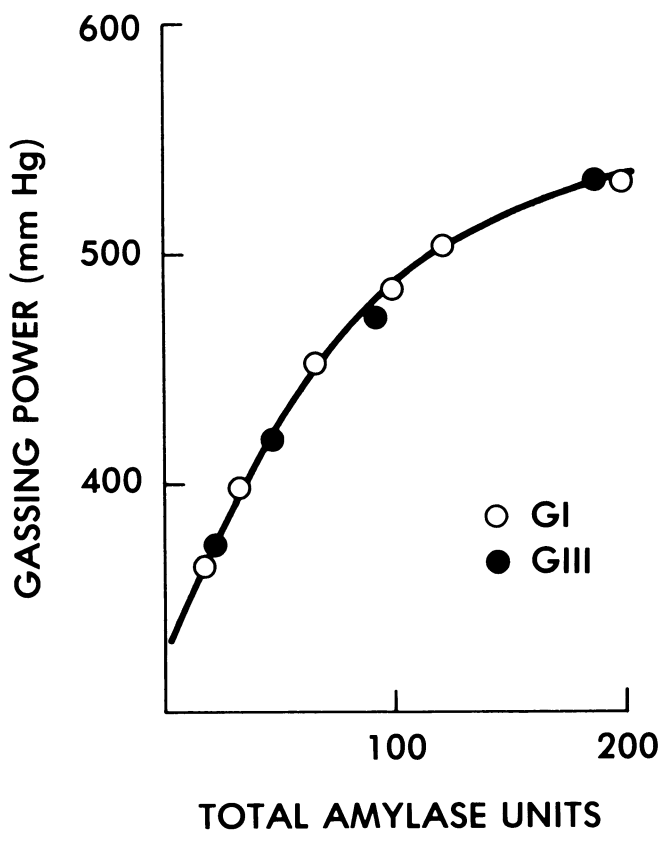

Fig. 10. Increase in the gassing power of a wheat flour with increasing addition of GI and GIII α-amylases from germinated wheat. One unit of α-amylase denotes the amount that hydrolyzes 1 mg of maltose/min × 10^{-3} at 37° C from reduced starch (Kruger and Tipples 1981).

group is more effective than the green GI α-amylase group in degrading starch granules. As suggested by Weselake and Hill (1983), different methods of isolation could impart different binding characteristics to the isoenzymes and thereby could explain the differences. Several other factors could explain the discrepancies, including the source material and manner in which the starch granules were prepared, temperature, pH, ratio of enzyme to substrate, etc. Caution must be used, therefore, in extrapolating the findings from a particular in vitro study to processes that actually occur in vivo during cereal germination.

The two purified α-amylase groups also were compared on two instruments that simulate a particular phase of the breadmaking process. Thus, the effect on gassing power reflects the relative abilities of the two groups to increase and sustain sugar levels during the fermentation process. Both components increased the gassing power to the same degree, with increasing concentration of enzyme, indicating that they have equal effectiveness and presumably identical action patterns in degrading damaged starch granules. The amylograph reflects the effectiveness of α-amylase to degrade starch granules during conditions that simulate the temperature cycle of the breadmaking process. Identical decreases in the amylograph peak viscosity were observed with increasing levels of enzymes. This was quite surprising, because the GI α-amylases have less heat stability than the GIII α-amylases (Marchylo et al 1976). They would consequently be inactivated sooner in the amylograph heating cycle and would have a correspondingly decreased effect in lowering the peak viscosity. One explanation of this phenomenon is that the high starch-water ratio provides added stability to the enzyme. Also, observations made on purified enzymes in dilute solutions cannot be translated ad hoc with respect to their expected behavior in an end-product process, such as breadmaking.

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