Modifications of Starch During Baking: Studied Through Reactivity With Amyloglucosidase

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ABSTRACT

Conditions that ensure starch hydrolysis by amyloglucosidase in a limited substrate system were worked out. Using these conditions, we evaluated the degree of access of the enzyme to starch molecules in different starchy materials. Raw starches of different botanical origins are hydrolyzed at different rates, but starches with limited branching hydrolyze more rapidly. A good example of this is a limit dextrin, which is more susceptible than its parent amylopectin. We also studied the effect of gelatinization on the enzymatic availability of starch. It was observed that damaged granules undergo amylolysis much more rapidly than do undamaged ones. Therefore, the extent of amylolysis in a given starch is governed by the degree of granule damage. Starch in bread is hydrolyzed more rapidly and extensively than is that in flour and dough, but no significant differences were found in conventional yeast fermentation between soft and durum wheat. On the other hand, bread obtained by acid fermentation initially undergoes slow amylolysis, although the final level reached is the same as in bread made from the same flour by conventional yeast fermentation.

The starch in cereals and cereal products cannot be considered an isolated system because once amylopectin and amylose are leached from damaged granules, they come into direct contact with proteins and other components such as fats and fiber. These components may be minor but are, nevertheless, relevant to the final texture of the product. Information on starch from isolated polymers takes no account of the integrity of the real system. This holds true even for granules, given the complexity of the structure and organization of amylose and amylopectin.

A less invasive approach is to evaluate the state and characteristics of starch by subjecting flour and bread to the action of amylolytic enzymes and monitoring the activity. Various investigations have been made using α-amylase (Björk et al 1986, Siljeström et al 1988) and amyloglucosidase (Lineback and Wongsrikasem 1980, Varriano-Marston et al 1980). However, such data need to be thoroughly examined to distinguish the effects due to the state of the starch molecule from the effects due to other factors that could affect enzyme activity. In fact, the assay of Varriano-Marston was on starch washed from bakery products, a process that could disrupt the integrity of the system.

In a research program on breads prepared using conventional and acid fermentations, we decided to study the characteristics of starch using amyloglucosidase (EC 3.2.1.3.), an enzyme that hydrolyzes α-1–4 and α-1–6 bonds and, therefore, both amylose and amylopectin. Because amyloglucosidase sequentially detaches all of the glucose units, it allows the entire polysaccharide breakdown to be monitored and used to define the polysaccharide condition. The only reaction product is glucose, a breakdown product that is more abundant, more specific, and more easily determined than those when other amylolytic enzymes are used.

Limited substrate conditions have been optimized to avoid misinterpretations: such analysis conditions have been applied not only to bread produced from *Triticum aestivum* or *T. durum* by both conventional yeast and acid fermentations, but also to model systems of amylose, amylopectin, and limit dextrin starches of various origin.

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MATERIALS AND METHODS

Materials

All chemicals were of analytical grade. Amyloglucosidase (EC 3.2.1.3.; 114.6 U/mg) from *Aspergillus niger*; α-amylase (EC 3.2.1.1.; 15 U/mg) from *A. oryzae*; β-amylase (EC 3.2.1.2.; 28 U/mg) from barley malt; and starches from wheat, maize, and rice were from Fluka. Amylose from maize (30% amylopectin), amylopectin from maize, and the kit for the assay of glucose were from Sigma.

Raw starch from wheat was used as such, and as a 0.4–1% suspension (w/v) heated at 60°C for 10 min or at 100°C for 10 min or 1 hr. The latter conditions produce complete gelatinization (Hill and Dronzek 1973).

Methods

Starch was determined according to Berry (1986), modifying the procedure by adding α-amylase from *A. oryzae* (1.2 U/10 mg starch) to amyloglucosidase from *A. niger* (30 U/10 mg starch) and incubating the mixture at 37°C for 15 min, followed by 50°C for 2 hr.

A limit dextrin was produced by the hydrolysis (37°C for 30 hr) of amylopectin (10 mg/ml) with β-amylase (0.1 g) in 100 ml of 0.04*M* Na-acetate buffer, pH 4.8. It was isolated by centrifuging the digested suspension at 4°C for 10 min at 12,100 × *g*.

Dough and bread were produced from commercial *T. aestivum* flour (flours 1 and 2) either by direct fermentation with compressed yeast or by acid fermentation with a *Lactobacillus brevis* starter added to flour 2.

Durum wheat doughs and bread were produced at the National Institute of Nutrition in Rome using the procedure described by Pasqui et al (1991). Four selected cultivars were used, and to ensure that the flours had the same protein content, grain from different regions were blended. The breadmaking quality, measured as bread volume according to Carcea et al (*in press*), decreased as follows: Capeiti (good, 675 cm^3), Grazia (657 cm^3), and Appulo and Latino (poor, 612 and 622 cm^3).

Our study was performed on a central part of the loaf, which was freeze-dried, ground, sieved (60 mesh), and stored at 4°C in vacuo. Water content was determined by measuring weight loss after heating in an oven at 110°C overnight.

Assay of amyloglucosidase activity. Starch (100 mg, dwb) was dispersed in water (25 ml) and, after stirring for 10 min, 1 ml was added to 2 ml of 0.1*M* Na-acetate buffer, pH 4.75; the solution

was heated to 60°C and then 0.25 mg of amyloglucosidase in 50 µl of buffer was added. After 10 min of incubation at 60°C, the reaction was stopped with 0.5 ml of 2.5*M* NaOH and the liberated glucose was determined enzymatically (glucose oxidase/peroxidase) with the Sigma kit. The reaction rate at time t was calculated as: rate = µg glucose$_{t2}$ – µg glucose$_{t1}$/($t_2 - t_1$). Total activity was taken as the glucose liberated after 30 min.

The flour, dough, and bread samples each contained 4 mg of starch, determined enzymatically. The assays were done in triplicate; the blank assay where 50 µl of buffer replaced the enzyme was done in duplicate.

RESULTS AND DISCUSSION

Optimization of Assay Conditions

Substrate. Figure 1 shows how amyloglucosidase activity is affected by the concentration of the substrate and the state of the starch. Increasing the substrate increases the liberated glucose, and it is clearly evident that in the conditions chosen for the assay (starch, 4 mg; enzyme, 0.25 mg) the enzyme is not saturated by the substrate, regardless of the starch gelatinization level. In the cited conditions, the governing factor for both rate of and total hydrolysis was the gelatinization level of the starch. This was very low in raw starch (A), moderate in bread (B) (Hoseney et al 1977, Pomeranz et al 1984), and complete in gelatinized starch (C). The final hydrolysis level is also given (note that as the total starch increases, this final level represents a lower percentage of the starting starch). Figure 2 shows how the hydrolysis rate decreased on prolonging the incubation; the residual activity after 30 min was very small.

Temperature. Under excess substrate conditions (fully gelatinized starch, 10 mg; enzyme, 0.05 mg) the highest activity was at 10 min of incubation at 60°C (*not shown*). With limited substrate, the modifications in activity depend on either the enzyme or the availability of the starch, and these two factors should be distinguished.

Activity measured at 50°C was less than at 60°C (Table I). In preheated samples (60°C), the activity, even when measured at 50°C, approached that measured at 60°C. Because the substrate was highly limited, this behavior depends on the presence, in the samples preheated at 60°C, of a larger amount of damaged granules, i.e., of available starch. For reference purposes, we have included the activity measured on fully gelatinized starch, e.g., that of the sample preheated at 100°C; in fact, this activity was far higher than in the other samples.

Access of Amyloglucosidase to Starch in Bread Products

The degradation kinetics of the examined materials are shown in Figure 2. A rapid phase, which in the conditions adopted takes about 5–10 min, was followed by a slow phase. Starch hydrolysis after 30 min of incubation was as follows: flour and dough, 6.4 and 7.1%, respectively; conventional yeast fermentation bread of flour 1, fresh and aged five days, 48.3 and 42.9%; conventional yeast fermentation and acid fermentation bread of flour 2, 36.2 and 37.6%; durum wheat breads of the cultivars Capeiti, Grazia, Appulo, and Latino, 45.5, 47.7, 48.2, and 44.8%, respectively. The recipe of *T. durum* bread includes the addition of a small amount of malt, which contains free sugars and amylolytic enzymes and a longer baking time. All these factors increase the glucose liberated and may flatten differences due to amyloglucosidase.

In flour and dough, both the reaction rate and the total amount of hydrolyzed starch were very low. In breads produced from cultivars of durum wheat, the initial hydrolysis rates differed slightly from those of other breads. The values of starch hydrolysis, namely rate and quantity hydrolyzed (i.e., starch availability to the enzyme) were equal in breads baked from a same flour regardless of the fermentation procedure. On the other hand, the breads made from the commercial flours, all baked by conventional yeast fermentation, produced different final hydrolysis values, although initially the hydrolysis occurred at the same rate. Indeed, final hydrolysis is influenced by the overall starch availability, and differences in commercial flours are possible.

Starches of Different Botanical Origin

To better understand the mechanisms of starch hydrolysis in bread, we considered starch isolated from wheat, maize, rice, potato, maize amylose, maize amylopectin, and a limit dextrin obtained from it by using β-amylase. Assays were done both on raw samples and after heating a 0.4% (w/v) water suspension for 10 min at 100°C. The results are reported in Figure 3. The gelatinization temperatures of the starches, from 52–63°C in wheat to 66–77°C in rice, are very close to that (60°C) used for incubation.

Raw substrates were poorly hydrolyzed. Their digestibility decreased in the following order: potato, rice, wheat, and maize. The limit dextrin was slightly more digested than amylopectin and amylose. There appeared to be no relation between digestibility in a raw starch, amylose content, and gelatinization temperature, but hydrolysis appeared positively related to the intrinsic viscosity of amylopectin (190, 145, 150, and 116 ml/g, respectively, for potato, rice, wheat, and maize starch) and negatively to the size distribution of amylopectin branches (5.5, 8.3, and 8.0 $DP_{av}15/DP_{av}45$, respectively, for potato, rice, and maize). The

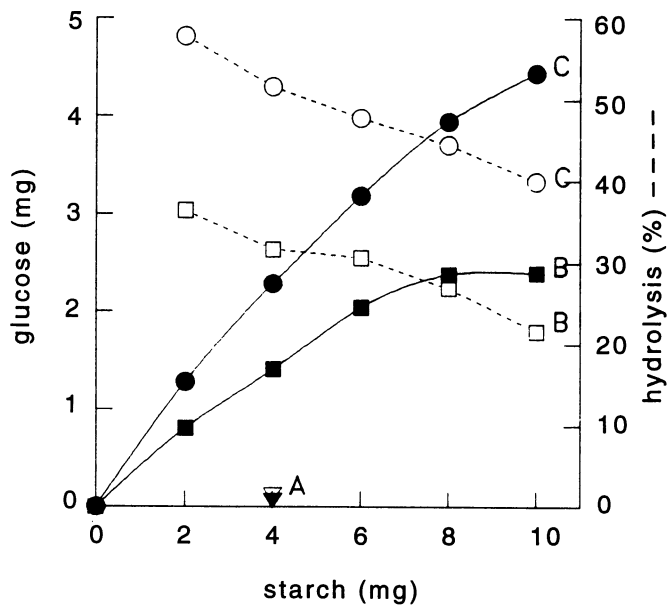

Fig. 1. Effect of substrate concentration on amyloglucosidase activity in 0.1*M* Na-acetate buffer, pH: 4.75, 10 min at 60°C. A = Raw wheat starch (▽, ▼), 0.05 mg enzyme. B = Conventional yeast fermentation bread (□, ■), 0.25 mg enzyme. C = gelatinized starch (○, ●), 0.05 mg enzyme. Solid symbols: glucose produced. Open symbols: percent hydrolysis.

TABLE I
Effect of Preheating on Amyloglucosidase Activity[a] as a Measure of Percent Hydrolyzed Starch

Condition	Starch A[b]	Starch B[b]	Bread[b]
Without preheating	1.4 ± 0.1	1.9 ± 0.1	23.4 ± 3.0
Substrate preheated 10 min at 60°C	2.3 ± 0.1	2.4 ± 0.1	21.2 ± 1.4
Substrate preheated 10 min at 100°C	n.d.[c]	37.7 ± 0.1	n.d.

[a] Preheated in the incubation buffer. Assay = enzyme: 0.25 mg, substrate: 4 mg of starch.
[b] Starch A incubated at 50°C, starch B at 60°C, and bread at 60°C.
[c] Not determined.

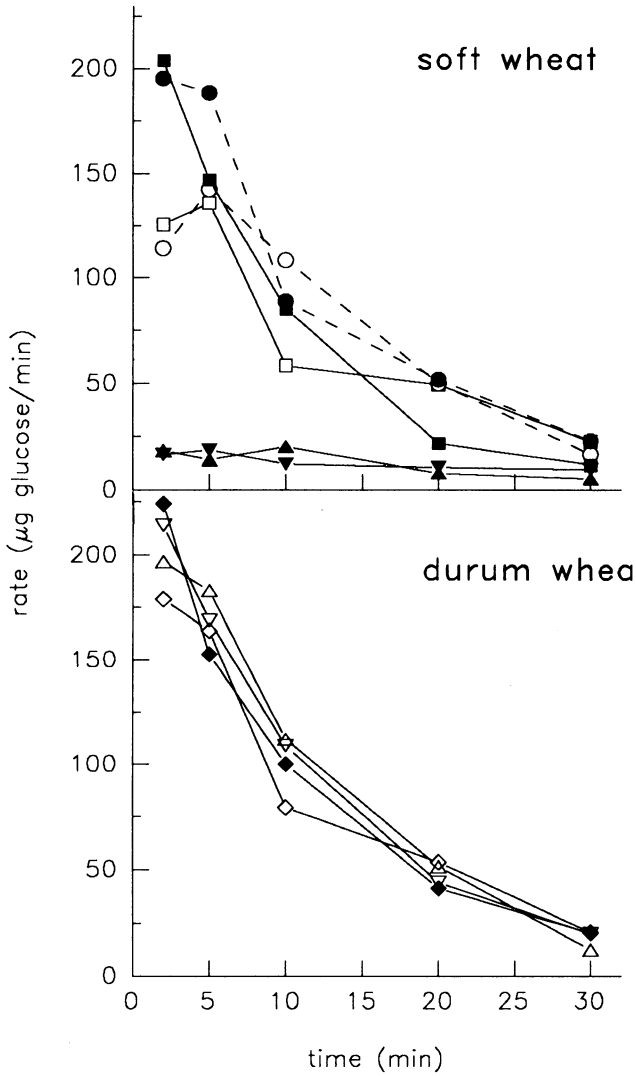

Fig. 2. Kinetics of amyloglucosidase activity on various starchy materials. Top: soft wheat flour (▲), dough (▼), bread from conventional yeast fermentation of flour 1, fresh (●) and aged five days (○), bread from conventional yeast fermentation of flour 2 (■), bread from acid fermentation of flour 2 (❑). Bottom: durum wheat bread from cultivars Capeiti (◆), Latino (◇), Grazia (∇), and Appulo (Δ). Substrate equivalent to 4 mg of starch, enzyme 0.25 mg. Other conditions as in Figure 1.

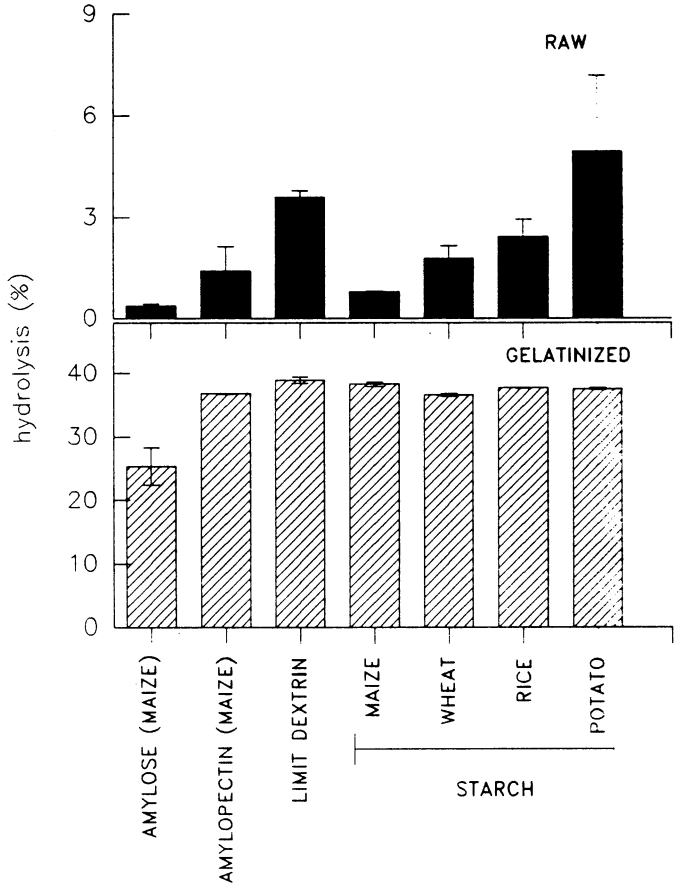

Fig. 3. Amyloglucosidase activity on purified starch and polysaccharides, raws (top) and after heating a 0.4% (w/v) water suspension 10 min at 100°C (bottom). Substrate 4 mg, enzyme 0.25 mg. Other conditions as in Figure 1.

data on starch characteristics are taken from Guilbot and Mercier (1985).

Gelatinized starches, amylopectin, and its limit dextrin gave similar hydrolysis percentages (between 36.6 and 38.9%) regardless of the origin of the starch. Amylose was less attacked by the enzyme (25.4% hydrolysis).

CONCLUSIONS

The different hydrolysis of the various substrates, which also depends on the preheating, can be attributed to the number of damaged granules, i.e., gelatinized starch, because it is well recognized that amylolytic enzymes have greater difficulty in acting on starch organized in whole granules (Colonna and Buléon 1992).

As was done for α-amylase acting on flour (Gibson et al 1992, Karkalas et al 1992), we have interpreted the rapid phase in amyloglucosidase activity as the enzyme acting on gelatinized starch, whereas in the slow phase the action of the enzyme is apparently conditioned by the undoing of granular structure. Compared to flour, the higher level of amylolysis in bread derives from the gelatinization of part of the granules during baking. If the gelatinized granules in our loaves are estimated from the final hydrolyzed starch, they should correspond to 36–49% of the total. Varriano-Marston et al (1980) report hydrolysis by amyloglucosidase as 70% in the bread, 30% in crust; however these authors measured starch washed out from bread baked under conditions different from ours.

The percent hydrolysis reached in flour and dough in our study, 6.4 and 7.1%, corresponded to that obtained on flour with α-amylase—5% by Björk et al (1986) and 8% by Gibson et al (1992)—and, with amyloglucosidase, 9% by Varriano-Marston et al (1980). Amyloglucosidase activity on flour and dough was both poor and similar, indicating that fermentation produced only minor modifications in the access of the enzyme to starch.

The different kinetics in the breads, prepared by conventional or acid fermentation from the same flour, indicated the effects of modifying breadmaking technology. The state of the starch and its interactions with other loaf constituents, particularly proteins, may play a role (Siljeström et al 1988), especially at the start of incubation. However, when the bread was incubated for a sufficient period, amylolysis reached a similar level in both flours: therefore, the dominating factor was the state of the starch molecule within the considered flour.

The lower hydrolysis found in stored bread, however, could be attributed to retrogradation that involved mainly amylose (Lineback and Rasper 1988).

Before gelatinization, each starch has its own individual structure and can be differentiated from other starches in the pattern of granule degradation: this was why the quantities that hydrolyze were smaller and different. Instead, gelatinized starches, regard-

less of their origin, are hydrolyzed in very similar amounts.

In ungelatinized material, preferential amylose exudation, as occurs at gelatinization, does not take place, whereas mechanical damage to granules is present and favors amylopectin leakage (Evers and Stevens 1985). No wonder, therefore, that there exists a parallel between the amount of starch hydrolyzed in raw starches by amyloglucosidase and the properties stemming from amylopectin. In fact, as reported by Colonna and Buléon (1992), potato starch, the most easily hydrolyzed, has shorter branches—the molecules are more disordered because the formation and coupling of helices and the organization of water at the outer surface are disfavored. This also increases resistance to shearing and produces higher viscosity. The same phenomena are present in limit dextrin, which has no branches at all. Instead, amylose is linear and has a high capacity to produce helices. Compared with amylopectin, amylose displays a larger number of interactions. This is probably why purified gelatinized amylose is less digested than amylopectin.

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