

Interactions of Protein and Starch Studied Through Amyloglucosidase Action

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ABSTRACT

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The access of amyloglucosidase to the carbohydrate molecule was taken as a measure of interactions occurring among starch, amylose, amylopectin, β -dextrin and purified gluten, gliadins, high molecular weight glutenin subunits, or bovine serum albumin when they were mixed together and gelatinized before digestion. The most relevant decrease in

liberated glucose, denoting coverage of some reaction sites for amyloglucosidase, occurred when gliadins were mixed with the carbohydrates. Other proteins were not as effective as gliadins. Amylopectin was not affected by any protein. Comparison of results allows some hypothesis to be formulated about the influence of structure on molecular interactions.

In all steps of breadmaking, proteins do not represent an isolated system; they are in close contact with starch, which is the main component and is still present in the form of granules. The macroscopic and rheological behavior indicate that during baking, starch interacts with gluten proteins (Dreese et al 1988; Martin et al 1991a,b). According to Martin et al (1991a,b), staling phenomena are also influenced by the interaction of protein with starch.

When starch and proteins are assayed separately, the integrity of the starch-protein system is overlooked. A less invasive approach in establishing the starch-protein interactions is to measure the availability of starch in the system to amylolytic enzymes before and after incubation with proteases. Holm et al (1985) and Bjorck et al (1986) reported increased availability after digestion with pepsin in wheat and wheat flour, respectively. Preliminary protein hydrolysis with pepsin restored the digestibility of acid fermentation bread by α -amylase to the values attained in conventional yeast fermentation bread (Siljeström et al 1988). The effects were attributed to interactions between starch and proteins (Siljeström et al 1988) in this case also. A similar approach to noninvasive starch modification is to compare amylolysis in products obtained from a same flour subjected to different processes. Starch in bread is hydrolyzed by amyloglucosidase more rapidly and extensively than it is in flour and dough. In acid fermentation, bread starch is less hydrolyzed by α -amylase (Siljeström et al 1988) and by amyloglucosidase than is starch in conventional yeast fermentation bread, indicating that a modified technology may affect starch availability (Eynard et al 1995).

Model systems containing starch and gluten proteins have been studied by various approaches. Dahle (1971) measured the ability of starch to bind iodine and proposed that interactions between starch and wheat flour proteins are of electrostatic nature. However, the effectiveness of this type of analysis for determining interactions with milk proteins has been questioned (Friedman 1995). Eliasson and Tjerneld (1990) reported that gluten proteins can associate with starch granules, whereas this does not occur for the water-soluble wheat proteins. The greatest association occurs at neutral pH, showing that the interactions probably are of hydrophobic nature. The interactions increase with protein concentration, and glutenins are absorbed preferentially (Eliasson 1993). Lindahl and Eliasson (1986) measured the rheological parameters of gels and proposed that the surface proteins of starch granules mediate gluten-starch

interaction. Greenwell and Schofield (1986) described a protein, friabilin, located at the surface of starch granules, which appears to mediate the interaction of the starch with the gluten proteins of the seed. Greenwell et al (1985) suggested that surface proteins in the starch granule may represent an obstacle to the access of amylolytic enzymes or may interact with them, modifying their surface distribution. However, his experiments using an ultramicroscopic technique did not find modifications in the amount of hydrolyzed starch.

Starch-protein interactions are of importance during the oven step in baking. During milling and baking, part of granules are damaged, and it is important to identify the interactions of the gluten proteins with the starch liberated from the damaged granules in order to understand the formation of the final product.

The present study examined whether and how the presence of proteins modified the activity of amyloglucosidase on the various substrates in model systems comprising starch or related polysaccharides and gluten proteins and compared the action of the various gluten proteins. The systems were heated to 100°C so as to approach the conditions existing in the dough during baking. The information obtained was developed to clarify the type of the interactions between the starch liberated from the granules and the protein and their possible technological application.

Amyloglucosidase was used instead of α - and β -amylase because it acts both on α -1-4 and α -1-6 bonds and it detaches one glucose unit at a time. This also makes the interpretation of results simpler. The behavior of amyloglucosidase on various starchy materials, and optimization of assay conditions, were described previously (Eynard et al 1995).

MATERIALS AND METHODS

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. Wheat starch and gliadins were supplied by Fluka. Maize amylose (30% amylopectin) and amylopectin, gluten, bovine serum albumin (BSA), and a diagnostic kit for glucose analysis were supplied by Sigma.

A β -limit dextrin was produced by hydrolyzing 1 g of amylopectin for 30 hr at 37°C with 0.1 g of β -amylase in 100 mL of 0.04M Na-acetate buffer, pH 4.8. The digest was cooled in ice and centrifuged 10 min at 12,000 \times g at 4°C. The pellet was frozen in liquid nitrogen, lyophilized, and stored in vacuo. The hydrolysis yield was calculated by determining the insoluble dextrin by dry weight. In 30-hr incubations, the pellet corresponded, by weight, to 38% of the original amylopectin, which indicates 62% hydrolysis. Prolonging the incubation until 48 hr did not change these values.

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Polysaccharides were determined by the procedure of Berry (1986) adapted as described by Eynard et al (1995).

The high molecular weight glutenin subunits (HMW-GS) were purified from a commercial soft wheat flour "00" with the procedure of Marchylo et al (1989). The product was not alkylated so as not to modify its natural charge. An electrophoretic control showed that no covalent intersubunit association occurred during heating the model systems. Gliadins were not affected.

Sample Preparation

Models were meant to reproduce the proportion of components in the bread system. The polysaccharides (starch, amylose, amylopectin, or β -dextrin) were either used as such or were added with gluten or gliadins or BSA, 10% of dry matter, or with HMW-GS, 1% of dry matter. Water, 60% (w/w, final), was added to the sample. The preparations were heated in a waterbath to 100°C for 10 min for gelatinization, followed by cooling in ice for 5 min. The preparations were then freeze-dried and ground to pass a 60-mesh screen. The water content of ground samples was determined by drying at 110°C overnight and was always <7% (w/w). Differential scanning calorimetry (DSC) studies have shown that if the polysaccharide is rapidly cooled after heating, as was done for our preparations, it remains in the amorphous state (Colonna and Bouleou 1992). β -Dextrin did not visibly merge as the other polysaccharides with 60% water (w/w). Therefore, 80% water (w/w) was considered. Samples without protein added were also assayed without gelatinization (i.e., with no heating).

As shown in a previous article on preheating starch for 10 min at 60°C, starch hydrolysis by amyloglucosidase had only negligible increase as compared to native starch, whereas consistent increase was obtained after preheating at 100°C (Eynard et al 1995).

Assay of Amyloglucosidase Activity

The assay conditions optimized by Eynard et al (1995) were used. The ground sample (100 mg, dwb), either native or gelatinized, was dispersed at room temperature in 25 mL of water and stirred for 10 min; 1 mL, corresponding to 4 mg of polysaccharide, was added with 2 mL of 0.1M Na-acetate buffer, pH 4.75, and heated at 60°C. The addition of 0.25 mg of amyloglucosidase in

50 μ L of buffer followed. The reaction was run at 60°C for different times and was stopped with 0.5 mL of 2.5M NaOH; the liberated glucose was determined enzymatically. Assays were performed in triplicate. Blanks were made in duplicate and 50 μ L of buffer replaced the enzyme. Under the conditions used the reaction develops under limit substrate conditions (Eynard et al 1995), allowing us to spot the modifications in the access of amyloglucosidase to starch.

Error bars do not appear in figures where standard deviation was small enough to be covered by the experimental point. Differences between curves mentioned in the text are always statistically significant, also where the experimental points and the error bars are not shown.

RESULTS

Amyloglucosidase Activity in the Absence of Proteins

Hydrolysis by amyloglucosidase of the various polysaccharides with no protein added was assayed on the native, unheated substrate and after gelatinizing by preheating at 100°C, as described above.

As shown by the hydrolysis kinetics in Fig. 1A and by the hydrolysis rates in Table I, native polysaccharides were digested very little. With heated substrates, a rapid step was visible in the first 10 min (Fig. 1B). It was followed by slowing down of the reaction. Upon prolonging the incubation, the degradation of some substrates stopped (native starch and amylose, gelatinized starch, amylopectin, and β -dextrin in 80% water), whereas for others (native amylopectin and gelatinized amylose) it continued at a reduced rate, which indicates that the enzyme was still active.

Gelatinization considerably improved the availability of the polysaccharides to the enzyme, consequent to modifications in their structure and aggregation (Fig. 1B vs. 1A). Under all conditions, amylose appeared less available to the enzyme, either from quantity digested or from digestion rate.

Amyloglucosidase Activity in the Presence of Protein

The possibility that the presence of proteins may modify the activity of amyloglucosidase was investigated using maltose as substrate. Assay conditions were optimized so as to reach substrate saturation and time linearity (not shown).

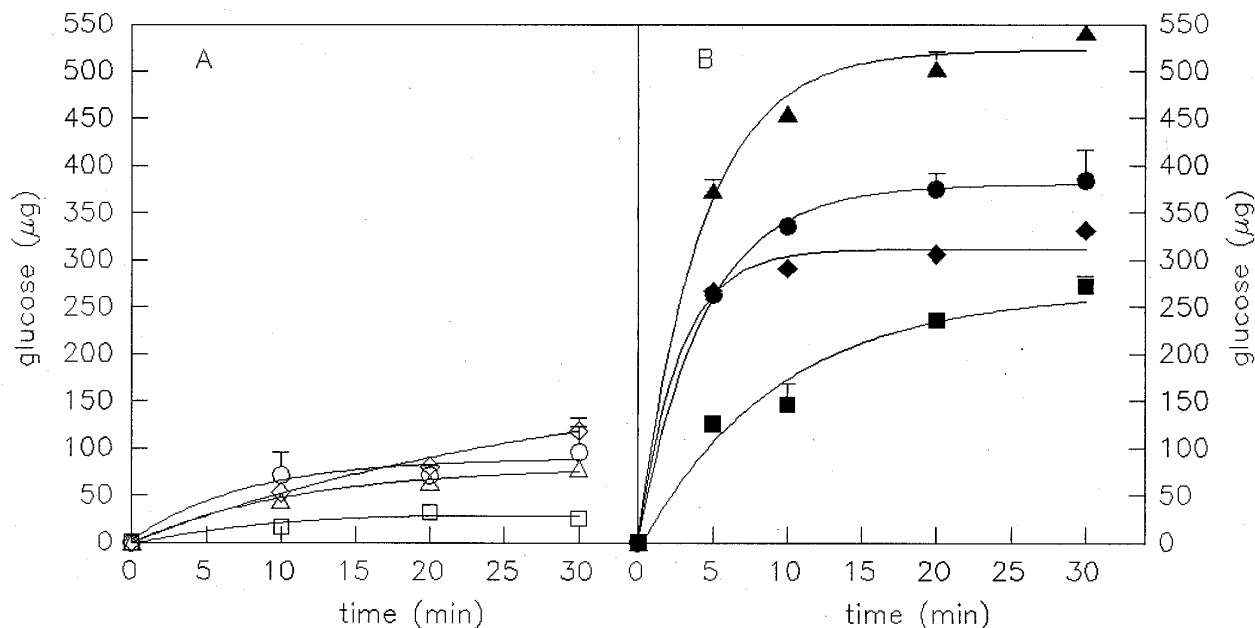


Fig. 1. Hydrolysis of native (A) and gelatinized (B) polysaccharides by amyloglucosidase in the absence of added protein. A, Native substrates (4 mg/mL) stirred in water for 10 min at room temperature and then incubated at 60°C with amyloglucosidase. B, Polysaccharides heated at 100°C for 10 min with 60 or 80% water (β -dextrin) were cooled in an ice bath, freeze-dried, and ground. Then they were stirred in water and incubated with amyloglucosidase as above. \circ , \bullet = starch; \square , \blacksquare = amylose; \diamond , \blacklozenge = amylopectin; Δ , \blacktriangle = β -dextrin. Ordinates: micrograms of glucose liberated by 1 mg of polysaccharide.

Amyloglucosidase incubated for 4 min in the conditions described with 10 mg of maltose produced 0.21 ± 0.02 mg of glucose. The activity developed linearly with time and was not modified (0.20 ± 0.03 mg of glucose) when the enzyme was preincubated for 10 min or for 30 min at 60°C with 0.4 mg of gluten, gliadins, or BSA, the same enzyme to protein ratio as in model systems. The added proteins remained in a dispersed state in the assay medium and were present during the assay of the glucose but did not interfere with it.

Hydrolysis Kinetics in Protein-Starch Systems

The results concerning the activity of amyloglucosidase on previously gelatinized polysaccharide substrates starch, amylopectin, amylose, and β -dextrin, respectively, in the presence of added protein, are grouped in Figs. 2–5 and in Tables I and II. The various proteins had different effects, indicating different interactions of the molecules on each polysaccharide.

The hydrolysis of starch was not affected by gluten or by BSA, while gliadins and HMW-GS both caused a similar decrease in the hydrolysis rate and in total hydrolyzed starch (Fig. 2, Tables I and II). Amylopectin had the same kinetics regardless of the protein added (Fig. 3, Tables I and II). The amylose systems were less accessible to amyloglucosidase than those with other substrates and all proteins, including BSA, moderately affected the initial rate. The final hydrolysis level underwent a moderate decrease due to gliadins, HMW-GS, and gluten (Fig. 4, Tables I and II). No effect of BSA was present. Gluten and HMW-GS caused some decrease in total digested β -dextrin (Fig. 5, Table II) but not in the initial rate (Table I). Gliadins conspicuously lowered both parameters.

DISCUSSION

The proteins that had an effect on the hydrolysis rate decreased the final hydrolysis level, indicating that part of the action sites for amyloglucosidase became inaccessible. Given the limited substrate, a decrease in reaction sites also produced the observed decrease in rate. In both proteins and polysaccharides, the interaction is governed by the structure and size of the molecules.

TABLE I
Hydrolysis Rate (μg glucose/min)^a

	Starch	Amylopectin	Amylose	β -Dextrin
Native	8.3 ± 0.2	5.6 ± 0.0	2.7 ± 0.0	5.8 ± 0.1
Gelatinized ^b	50 ± 2	49 ± 3	23 ± 0.4	69 ± 3
Gelatinized with added gliadin	39 ± 2	46 ± 3	15 ± 3	43 ± 3
Gluten	55 ± 1	50 ± 5	15 ± 0	72 ± 7
HMW-GS	42 ± 2	46 ± 2	19 ± 0	72 ± 4
BSA	57 ± 4	51 ± 3	15 ± 0	71 ± 5

^a Hydrolysis rate was calculated as first derivative of the hydrolysis curve in the initial 5 min.

^b Starch, amylopectin and amylose were heated in the presence of 60% water; β -dextrin with 80% water.

TABLE II
Final Hydrolysis^a

	Starch ^b	Amylopectin ^b	Amylose ^b	β -dextrin ^c
Gliadin	80 ± 7	94 ± 2	74 ± 2	53 ± 6
Gluten	99 ± 7	96 ± 2	89 ± 7	84 ± 8
HMW-GS	79 ± 11	105 ± 4	80 ± 5	87 ± 8
BSA	99 ± 11	99 ± 8	99 ± 11	101 ± 2

^a Hydrolysis values after 30 min of incubation are expressed as percentage of hydrolysis of the gelatinized polysaccharide without additions. The standard deviation is calculated by summing the errors on the activity measured without and with protein added. Therefore high values may result.

^b Gelatinized in the presence of 60% water.

^c Gelatinized in the presence of 80% water.

Gliadins were most efficient. They are monomers, more flexible, larger in size, and may adjust better to the polysaccharide than HMW-GS. The effect of gliadin was significant on β -dextrin.

The HMW-GS and gluten had smaller effects. The interaction between gliadins and glutenins existing in gluten probably reduced its interactive capacities.

Lack of effect of protein addition on amyloglucosidase activity assayed with maltose as substrate indicates that the activity modifications observed in model systems having a polysaccharide substrate are not due to an effect on the enzyme. They depend rather on the interaction of the protein with the polysaccharide—the protein may act as an obstacle or impede enzyme action. Continuing activity, on several substrates, indicates that the enzyme was not deactivated upon long incubation.

The slow and continuing degradation of the native amylopectin and β -dextrin may be explained considering that hydrolysis of branched substrates involves a preliminary disentanglement of the chains into single chains (Colonna and Buleon 1992).

After heating, β -dextrins, which are smaller and have much shorter chains, were most susceptible to protein addition, whereas amylopectin was not visibly affected. Its branched structure and the helical structure of branches most likely hindered close contact with the proteins. Limited interaction with amylopectin has also been reported for other proteins. Evaluation of glass transition of 1:1 mixtures of amylopectin and casein using thermal methods of analysis seems to support the idea that the two polymers are not miscible (Kalichevsky and Blanshard 1992). This may account for the lack of influence of some starches in the coagulation of milk proteins (Muncy and Olsen 1988) and yogurt (Friedman 1995). The presence of amylopectin in starch, the hydrolysis of which is unaffected by the proteins, is the most likely for the lack of effect of proteins on starch hydrolysis. Amylose reportedly forms aggregates with gliadins while amylopectin does not (Friedman 1995).

The limited amyloglucosidase activity on amylose confirms our previous results (Eynard et al 1995). It may be indication, as well as the scarce effect of proteins on the degradation, of the presence of a structured phase in this substrate after the heating-cooling treatment (Colonna and Buleon 1992).

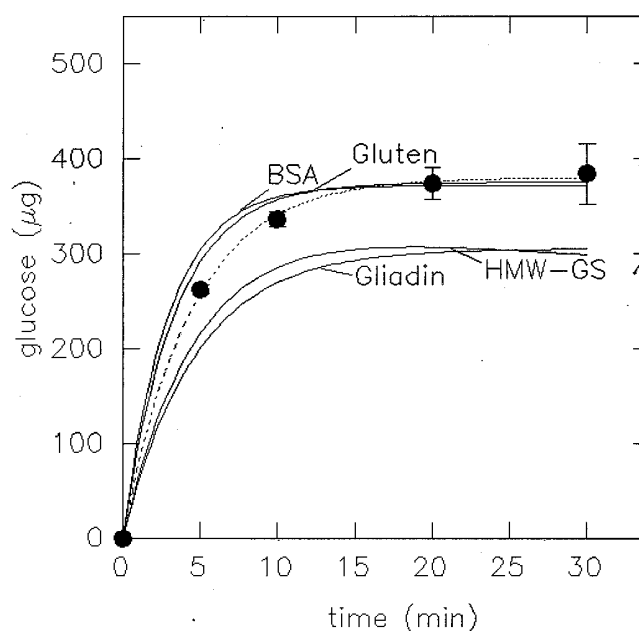


Fig. 2. Effect of proteins on the hydrolysis by amyloglucosidase of starch (solid line). Gelatinization in the presence of proteins and assays were as in Fig. 1B. For clarity reasons, the experimental points are given only in the sample with no additions (dash line). HMW-GS = high molecular weight glutenin subunits.

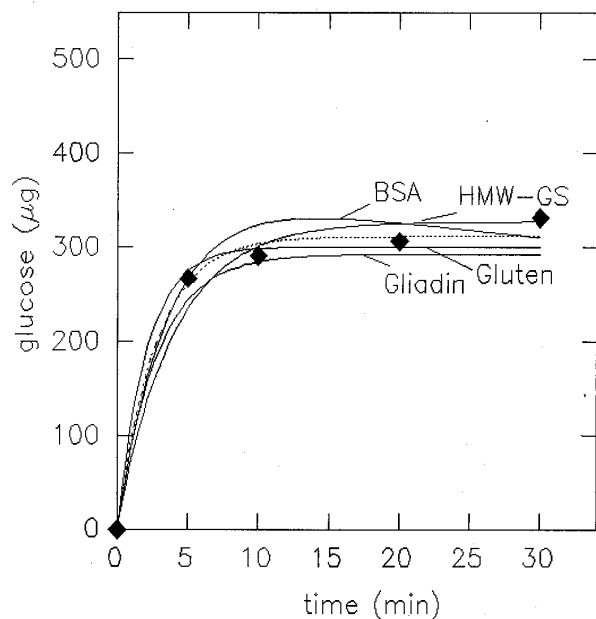


Fig. 3. Effect of proteins on hydrolysis of amylopectin by amyloglucosidase.

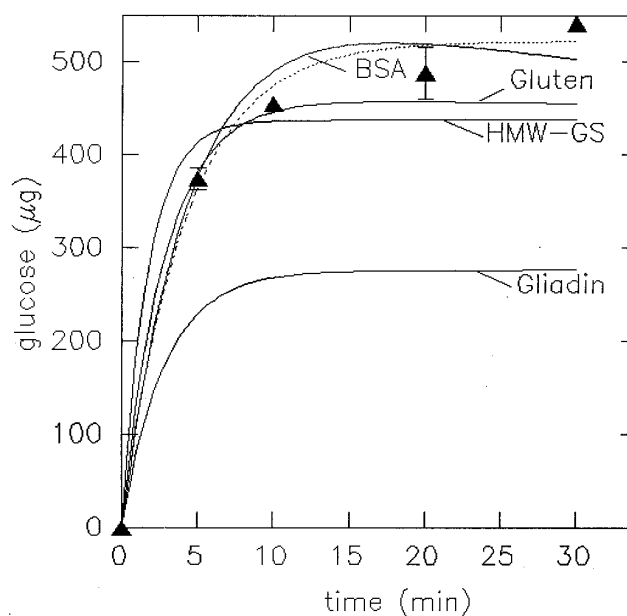


Fig. 5. Effect of proteins on hydrolysis by amyloglucosidase of β -dextrin gelatinized in 80% water (final, w/w).

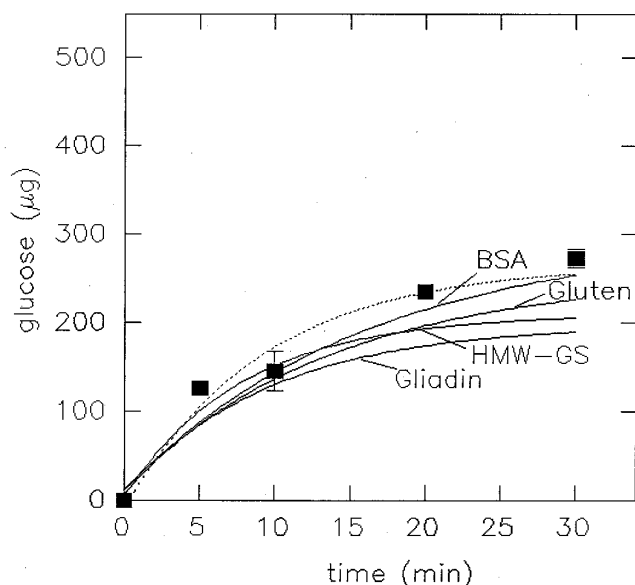


Fig. 4. Effect of proteins on hydrolysis of amylose by amyloglucosidase.

Using DSC, Eliasson (1983) has shown that gluten, in a system with starch and limited water, competes with starch for water available for gelatinization. The protein thus hinders sugar destructuring; this is one possibility whereby, under our conditions, the effect of amyloglucosidase on the sugar is reduced.

As shown by the experiments (Fig. 1A), decreased accessibility to the enzyme survived the contact with high amounts of water during incubation with amyloglucosidase. This indicates that water availability was not important if it occurred below the transition temperature of the polysaccharide. It also indicates that the interaction established at 100°C is rather strong and continues to operate during incubation with amyloglucosidase. The problem whether it has or not covalent nature has to be worked out.

One possible alternative is that the interactions between polysaccharide and protein were not mediated by available water and depended only on molecular specificity. Indeed, BSA had no effect on amyloglucosidase activity. According to Harding et al (1993), BSA does not interact with wheat polysaccharides on heating.

The temperature dependence of the effects observed stresses the importance of the structural transition related to gelatinization.

Most granules should have melted during the heating treatment. Therefore, the interaction between the protein and the polysaccharides evidenced in the present study is distinct from association with granules, which is implied in the experiments of Eliasson and Tjerneld (1990), Eliasson (1993), and Petrofsky and Hosney (1995) and is supposed to be mediated by the surface protein of the granules (Lindahl and Eliasson 1986, Greenwell and Schofield 1986).

Protein-starch interactions are used in the food industry to improve the structural properties of food products (Friedman 1995).

The heated systems we used containing starch, protein, and limited water, interpret reasonably well what happens in dough during the heating phase after fermentation: they evidence possible interactions between starch and proteins and how they depend on temperature. It appears that starch gelatinization as shown by its availability to the enzymes is influenced by protein and presumably also by the quality of protein (i.e., by the flour quality). Studies in this line are in progress.

ACKNOWLEDGMENT

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