

A Mechanism of Bread Firming. II. Role of Starch Hydrolyzing Enzymes¹

M. L. MARTIN² and R. C. HOSENEY³

ABSTRACT

Cereal Chem. 68(5):503-507

High levels (100 SKB units per loaf) of bacterial α -amylase stopped bread from firming during five days of storage. Supplementing bread with malted barley flour did not decrease the firming rate. Bread supplemented with either fungal or bacterial α -amylase contained larger quantities of residual dextrins (degree of polymerization 3-9), whereas bread supplemented with malted barley flour contained only small quantities of those dextrins. β -Amylase was effective in reducing bread firmness; however, very high levels of supplementation did not completely

stop the bread-firming process. Bread supplemented with pullulanase firmed at a faster rate than did the unsupplemented control bread. Dextrins of a particular size are presumably responsible for the antifirming effect. The suggested mechanism of bread firming maintains that cross-links between protein fibrils and starch granule remnants result in bread firming. Dextrins of a particular size interfere with these cross-links (hydrogen bonds) between starch and protein.

The mechanism of bread firming has not been well established. For many years, the theory that amylopectin recrystallization within starch granules resulted in bread firming was generally accepted as fact. It is well known that bacterial α -amylases decrease the rate of bread firming (Conn et al 1950, Schultz et al 1952). During baking, amylases partially hydrolyze starch molecules to small dextrins. Several authors have suggested possible mechanisms by which amylases function to decrease bread firmness. Schultz et al (1952) suggested that the increase in low molecular weight dextrins produced by bacterial α -amylase resulted in a slower rate of retrogradation. Zobel and Senti (1959) used X-ray diffraction and showed that recrystallization increased in bread supplemented with bacterial α -amylase. They proposed that the dextrins disrupted the continuity of the starch network and reduced its rigidity.

Action of Amylases

The initial action of α -amylase may be essentially a random attack, with the enzyme having equal affinity for all α -(1-4) glycosidic linkages except those adjacent to the two ends of the starch chain and those in the vicinity of branch points. Once the enzyme complexes with the starch molecule and the initial cleavage has been made, the enzyme may remain with one fragment and produce one or more cuts before dissociating and moving to another substrate molecule (Robyt and French 1970).

β -Amylase attacks the next-to-the-last α -(1-4) glycosidic bond from the nonreducing end of a starch molecule to release maltose; α -1-6 branch points block the action of β -amylase. The enzyme

repeatedly cleaves a starch molecule chain before dissociating (Robyt 1984).

The amylose-lipid complex was shown to be an obstacle to starch hydrolysis with glucoamylase (Abe et al 1988). The initial velocity of reducing end-groups released by the action of α -amylase (*Bacillus subtilis*) was lower in the presence of 10% monoglyceride. Starch granules were less swollen in the presence of a monoglyceride, which may have decreased the rate of hydrolysis (Van Lonkhuisen and Blankestijn 1976).

Window of Amylase Activity

Several workers have determined the temperature profile of bread baked in a conventional oven (Walden 1955, Audidier 1968). Walden (1955) extracted α -amylase from malted barley and measured wheat starch conversion as a function of temperature during baking. Below 55°C, starch was not gelatinized and amylase activity was insignificant. Above 95°C, thermal denaturation of malt amylase was complete. Starch was rapidly attacked over the approximate temperature range of 58-78°C. Above 78°C, the rate of conversion declined because of enzyme denaturation. Increased quantities of α -amylase produced a decrease in the average degree of polymerization (DP) of the dextrins produced. Above a certain level, additional enzyme did not change the size of the dextrins produced. The substrate affinity of the enzyme for the smaller dextrins may have been unfavorable. The rate of enzyme denaturation was not dependent on quantity of α -amylase present. Walden also measured starch conversion to maltose by β -amylases. Denaturation occurred over the temperature range of 54-74°C. A complementary effect of α - and β -amylases acting jointly was reported. Used together, both enzymes produced 26.7% conversion of wheat starch; however, when used singly, α - and β -amylases produced 6.1 and 6.2%, respectively. Average dextrin chain length was reduced from 60 when α -amylase acted alone to 23 when a combination of α - and β -amylases was present. In malted commercial flours, β -amylase was present in sufficient quantities that the principal product of starch conversion during baking was maltose. When α -amylase supplementation

¹Contribution No. 90-592-J. Kansas Agricultural Experiment Station, Manhattan, KS 66506. Presented at the AACC 74th Annual Meeting, Washington DC, November 1989.

²Present address: Nordreco AB, Bjuv, Sweden.

³Graduate research assistant and professor, respectively. Department of Grain Science and Industry, Manhattan, KS 66506-2201.

was substantially increased without an increase in β -amylase, large quantities of dextrans were produced (Walden 1955).

Effect of Malted Barley Flour on Bread Firming

In the United States, 0.1–0.5% malted barley flour is normally added to flours used in yeast-leavened breads. Johnson and Miller (1948) showed that up to 6% added malted barley flour did not have a detrimental effect on bread quality or reduce bread firmness. In apparent contrast, Ponte et al (1963) baked bread containing 0.1 and 0.2% malted barley flour and measured firmness with a Baker compressimeter. Increased levels of malted barley flour lowered the firming rate.

The present study was undertaken to investigate the effect of starch hydrolysis on bread firming and incorporate the information obtained into a proposed mechanism of bread firming.

MATERIALS AND METHODS

Flours used were donated by Cargill (Wichita, KS). The flours had protein contents of 11.5–12.1% (14%, mb). All flours used firmed at essentially the same rate. Bacterial α -amylase was Dex-lo P from Gist-Brocades (King of Prussia, PA). β -Amylase type I-B from sweet potato, fungal α -amylase from *Aspergillus oryzae*, and pullulanase from *Enterobacter aerogenes* were purchased from Sigma Chemical Co. (St. Louis, MO). The monoglyceride was AM 341 from Grindsted Products, Inc. (Industrial Airport, KS). Other ingredients were as specified by AACC method 10-09 (AACC 1983).

Bread was baked according to the standard pup-loaf procedure of AACC method 10-09 (AACC 1983). Fermentation time was 180 min. Fermipan instant yeast (0.76 g) was used instead of compressed yeast, and malted barley flour was used in place of malt syrup.

Bacterial α -amylase was in powdered form, and the stated activity level was 55,000 SKB/g. It was dispersed in water to facilitate supplementation in bread. Dilution of the enzyme to 50 SKB/0.01 ml did not affect its activity, but dilution to 50 SKB/ml destroyed its activity. β -Amylase, 27,930 Sigma units (SU)/ml, was used as received or diluted to 1,000 SU/ml. (SU for β -amylase is defined as follows: One unit will liberate 1.0 mg of maltose from starch in 3 min at pH 4.8 at 20°C.) Dilution did not affect the activity of β -amylase or fungal α -amylase.

Bread was stored in polyethylene bags at ambient temperature for five days unless otherwise noted. Firmness was measured as described in the companion article (Martin et al 1991).

The high-performance liquid chromatography (HPLC) system was a Varian (Palo Alto, CA) Model 5000 equipped with a 20- μ l automatic injection loop. Detection was with a refractive index detector (ERMA Optical Works). The equipment had a Hewlett Packard Model 3392A integrator. A CH-30 column heater (Anspec Co. Inc., Milwaukee, WI) that maintained 85°C was

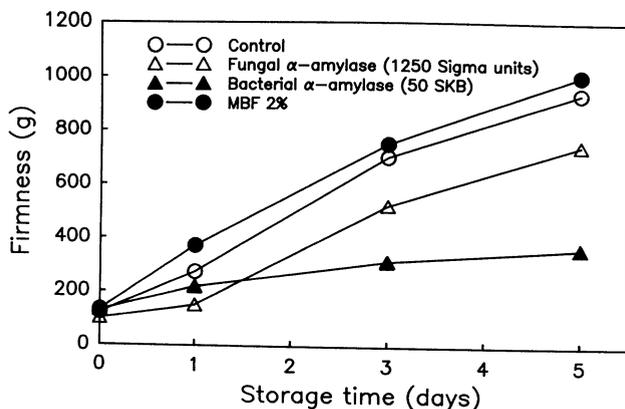


Fig. 1. Effect of malted barley flour (MBF), bacterial α -amylase, and fungal α -amylase on bread firmness.

used to detect oligosaccharides DP 2–9.

Residual maltose was separated from bread crumb and quantified by HPLC according to AACC method 80-04 (AACC 1983). A Bio-Sil amino 5S column, 250 \times 4mm, equipped with an Amino 5S guard column (Bio-Rad Laboratories, New York), and packed with a 5- μ m amino-bonded silica material, was used to separate glucose, fructose, lactose, maltose, and sucrose at room temperature.

After the bread was stored for five days in polyethylene bags, the crust was removed and the moist crumb was ground in a coffee grinder. Crumb moisture was determined by AACC method 44-18 (AACC 1983). A 10-g sample of bread crumb was placed in a 250-ml centrifuge bottle, and 100 ml of distilled water was added. The sample-water slurry was stirred with a magnetic stirrer for 25 min at room temperature. The sample was then centrifuged at 1,000 \times g for 10 min. Supernatant was filtered twice—first through a Whatman No. 2 filter paper and then through a No. 5 filter—and refrigerated. Within the next 48 hr, the supernatant (10 ml) was placed in a 50-ml volumetric flask, diluted to volume with acetonitrile, and allowed to warm to room temperature. Samples were filtered through a 5- μ m filter (Millipore) before injection onto the HPLC column. The mobile phase was a mixture of acetonitrile and water (80:20). An eluant flow rate of 1.0 ml/min was used. Column temperature was ambient, the refractive index detector was set at 35°C, and chart speed was 1 cm/min.

An Aminex HPX-42A column (Bio-Rad) packed with 4% cross-linked cation exchange resin in the silver form and equipped with a carbohydrate deashing guard column was used. The mobile phase was 100% distilled water. Eluant flow rate was 0.5 ml/min. The extraction procedure for analysis of dextrin chain length after debranching is similar to the procedure described above. Before dilution with acetonitrile, the supernatant was slurried with ethanol (40 ml), filtered, evaporated, and rehydrated. Pullulanase was used for debranching when specified.

RESULTS AND DISCUSSION

Effect of Monoglyceride on Thermal Stability of Amylases

Surfactants have been shown to stabilize and denature proteins. Denaturation temperatures of bacterial α - and β -amylases were measured by differential scanning calorimetry (DSC). DSC scans of bacterial α -amylase showed two endotherms with onset temperatures of 75 and 95°C. β -Amylase (Sigma) suspended in a 2.3M solution of $(\text{NH}_4)_2\text{SO}_4$ showed a single endothermic peak with onset at 74°C. Addition of monoglyceride to α - or β -amylase did not shift the onset temperature of the endotherms. These results indicated that the monoglyceride did not function by affecting the thermal stability of the amylases.

To determine whether monoglyceride affected the activity of β -amylase by other means, residual maltose in bread crumb was measured. Addition of monoglyceride to the bread formula did not affect the quantity of residual maltose present in the bread (2.8%, db). However, bread crumb supplemented with monoglyceride (1%) and β -amylase (50 SU per gram of starch) had less residual maltose (3.4%) than did bread crumb with β -amylase only (4.5%). Therefore, in a system with high levels of β -amylase, monoglyceride appeared to reduce β -amylase activity.

To further characterize the functionality of monoglyceride, bacterial α -amylase was added to bread supplemented with β -amylase and monoglyceride. Residual maltose content of bread crumb indicated that bacterial α -amylase overcame the effect of monoglyceride on β -amylase activity.

Role of α -Amylases in Bread Firming

To further understand the mechanism of bread firming, breads supplemented with bacterial α -amylase, fungal α -amylase, or malted barley flour were compared (Fig. 1). Bread baked with a high level of bacterial α -amylase did not firm during five days of storage. Bread baked with 1,250 SU of fungal α -amylase firmed at a moderate rate. However, bread supplemented with malted barley flour (2%) firmed at essentially the same rate as did the control bread.

Window of Amylase Activity

A window of amylase activity exists between the temperature at which starch begins to swell and the temperature at which amylases are denatured. During this window of activity, amylases degrade starch. To confirm that a window of activity existed during baking, dextrans (DP 3-9) in water solubles extracted from dough and bread crumb were measured by HPLC. Doughs prepared with or without bacterial α -amylase (50 SKB units/loaf) and fermented for 5 hr did not contain any measurable amount of dextrans between DP 3 and 9. After baking, no dextrans between DP 3 and 9 were found in the water-soluble extract of bread (control formula). However, dextrans in the DP 3-9 range were detected in bread baked from flour supplemented with bacterial α -amylase. A profile of the dextrans extracted from bread crumb is shown in Figure 2. Clearly, a window for activity of amylases on starch existed during baking.

Effect of β -Amylase

Firmness and residual maltose contents of bread made with flour supplemented with various levels of β -amylase are shown in Table I. Addition of 1,000 SU of β -amylase per gram of starch resulted in 7.1% residual maltose, whereas 50 SU of β -amylase resulted in 4.2% residual maltose. Bread made from flour supplemented with 50-1,000 SU of β -amylase were similar in firmness after five days of storage.

Flour Plus α -Amylase

The effect of bacterial α -amylase at 10, 20, and 50 SKB units per loaf on bread firmness is shown in Figure 3. Bread baked from flour supplemented with 50 SKB units of bacterial α -amylase firmed at a rate of 81 g/day. Bacterial α -amylase at 100 SKB units per loaf effectively inhibited bread firming. Bacterial α -amylase is more thermostable than is cereal α -amylase; therefore, large quantities of cereal α -amylase were used to attempt to overcome the shorter window of activity. Bread baked from flour with either 1 or 2% malted barley flour added to the formula firmed at a faster rate than did bread baked without malt supplement (Fig. 4). The addition of 10% malted barley flour

(about 100 times the normal level of addition) still firmed (data not shown). Therefore, it appears that the antifirming mechanism of bacterial α -amylase involved more than a higher degree of dextrinization of starch during a longer window of activity.

In an excess water system and with starch as a substrate, the method of attack of α -amylase has been described as essentially random cleavage of α -(1-4) glycosidic linkages. However, in a limited water system such as bread, the degradation of the starch would not be randomized throughout the granule. With limited swelling, the enzyme activity would be concentrated on the outside or near the surface of the granule. The enzyme is large and does not diffuse rapidly into a partially swollen starch granule. The substrate most easily hydrolyzed might be the starch molecules that had been leached or partially leached from starch granules.

Analysis of Residual Dextrans in Bread

Bread supplemented with bacterial α -amylase (100 SKB units per loaf) did not firm during five days of storage. In contrast, bread supplemented with malted barley flour firmed at a faster rate than did unsupplemented bread. Based on the inconsistency of these results and the "known" antifirming effects of α -amylase, we sought to understand why malted barley flour did not have an antifirming effect. The residual dextrans of DP 3-9 produced by the enzyme systems were studied. Profiles of dextrans extracted from bread supplemented with bacterial α -amylase and malted barley flour are shown in Figures 5 and 6, respectively. Dextrans of DP 3-7 were found in the extract from bread supplemented with bacterial α -amylase. No extractable dextrans of DP 3-9 were found in bread supplemented with 2% malted barley flour. The quantity of dextrans in the void volume indicated that the dextrans produced by the malted barley flour were larger than DP 9. Dextrans of DP 3-9 also were present in bread supplemented with fungal α -amylase (data not shown).

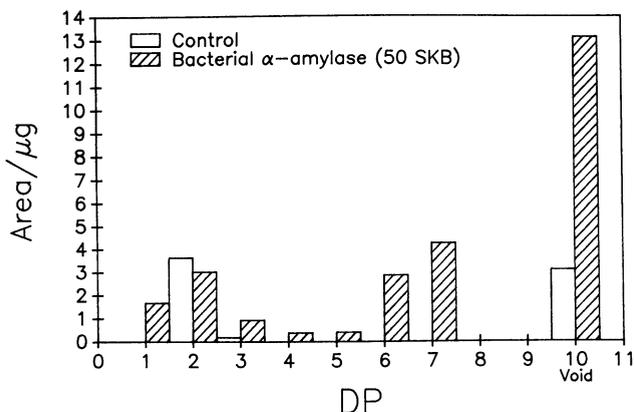


Fig. 2. Profile of water-soluble dextrans extracted from unsupplemented bread crumb and bread supplemented with bacterial α -amylase. DP = degree of polymerization.

TABLE I
Effect of β -Amylase on Residual Maltose and Bread Firmness

| Treatment | Firmness on Day 5 (g) | Residual Maltose (% db) |
|---|-----------------------|----------------------------|
| Flour | 1,210 | 2.6 \pm 0.2 ^a |
| + β -amylase (50 SU) ^b | 1,020 | 4.2 \pm 0.2 |
| + β -amylase (200 SU) | 965 | 5.4 |
| + β -amylase (1,000 SU) | 1,015 | 7.1 |

^aSD, n = 3.

^bSigma units: One unit will liberate 1 mg of maltose from starch in 3 min at pH 4.8 at 20°C.

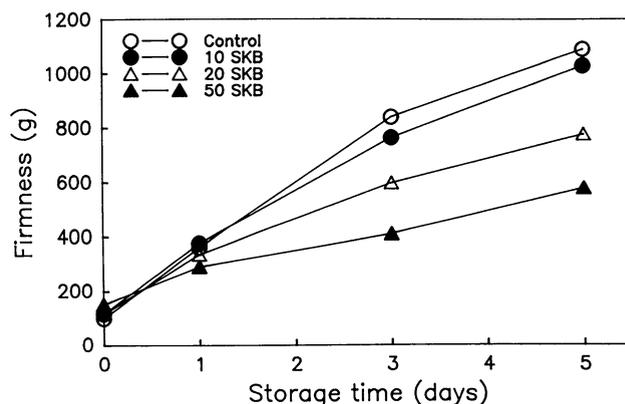


Fig. 3. Effect of bacterial α -amylase on bread firmness.

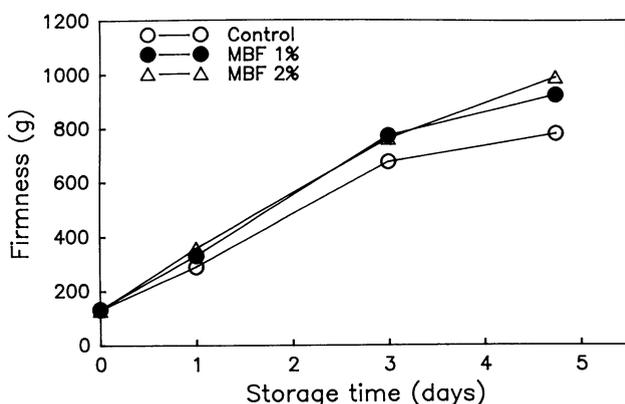


Fig. 4. Effect of malted barley flour (MBF) on bread firmness.

Relevance of a Debranching Enzyme

α -Amylase produces branched dextrans of random size. If α - and β -amylases and a debranching enzyme were all active in a bread system, the lack of residual dextrans in a flour + malt system would be explained. Results would depend on the relative heat stability of the different enzymes.

Pullulan was hydrolyzed with pullulanase. HPLC analysis showed the presence of dextrans of many sizes. Thus, pullulanase attack was essentially random, and the dextrans produced were resolved on the HPLC column.

Malted barley flour was added to pullulan to determine if malted barley flour contained a debranching enzyme specific for α -(1-6) glycosidic linkages. After incubation at 30°C for 24 hr, HPLC of the water-soluble extracts did not show an increase in malto-

triose. Therefore, the supposition that the malted barley flour contained a debranching enzyme was not supported.

Pullulanase was added to the water-soluble extract from bread crumb with malted barley flour added. The HPLC column that was used resolved low molecular weight dextrans (DP less than 9). Ethanol was used to precipitate the higher molecular weight dextrans from the water-soluble extract of bread. HPLC analysis showed that residual dextrans of DP 5 and 6 increased after ethanol precipitation. The ethanol-soluble fraction after rehydration in water was hydrolyzed with pullulanase (1%). This had only a minor effect on the profile of dextrans. Dextrans greater than DP 8 were eliminated and those of DP 5 increased. Quantities of other dextrans were essentially unchanged (Fig. 7). The lack of a significant increase in low molecular weight dextrans indicated that most of the water-soluble dextrans of less than DP 8 were not branched. Therefore, the antifirming effect of bacterial α -amylase vs. the lack of effect of malted barley flour might be related to the size of the dextrans produced and not the presence of branched dextrans.

Effect of Pullulanase on Bread Firming

Bread supplemented with pullulanase (10 SU) firmed at a faster rate than did unsupplemented bread. The firming profile of bread supplemented with pullulanase is shown in Figure 8. Debranching amylopectin yielded a wide distribution of dextrin chain lengths from 12 to more than 90 glucose units (Kobayashi et al 1986). Based on the above, it appears that high molecular weight dextrans do not have an antifirming effect on bread crumb. Instead, the high DP dextrans may entangle and/or form a hydrogen bond with protein fibrils, thus effectively cross-linking the gluten. Consequently, the firming rate is increased.

A Model of Bread Firming

The fact that bread supplemented with malted barley flour (2%) did not contain low molecular weight dextrans and did not retard firming, whereas bread supplemented with either fungal or bacterial α -amylase contained low molecular weight dextrans and retarded firming, suggests that the low molecular weight dextrans indeed have antifirming properties. It appears that the different sources of α -amylase produce fragments of different sizes. Dextrans of an intermediate size are effective in decreasing bread firmness.

These results can be applied to the mechanism of bread firming presented in the companion article (Martin et al 1991). A model of the mechanism of bread firming and the effect of dextrans on the mechanism are presented in Figure 9. Because maltose is a small molecule relative to larger dextrans, it may diffuse away from the interface between starch and protein. If maltose molecules are not at the interface, interference with the entanglements between starch and protein or protein/protein does not occur. Large dextrans produced by malted barley flour and pullulanase enhance the rate of bread firming. The most reasonable explanation at present is that the larger dextrans act to cross-

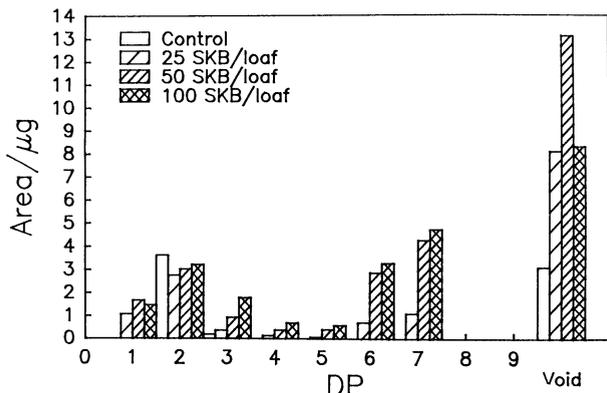


Fig. 5. Profile of dextrans extracted from bread supplemented with bacterial α -amylase. DP = degree of polymerization.

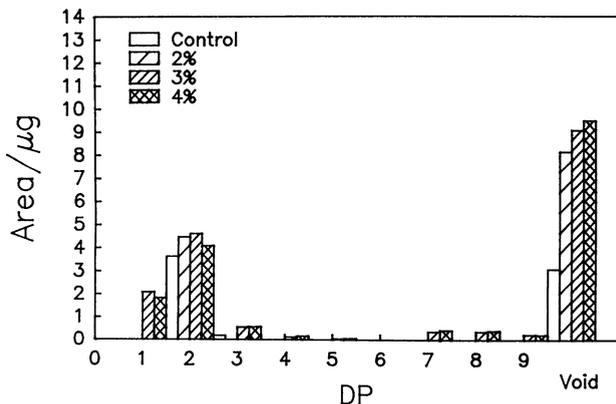


Fig. 6. Profile of dextrans extracted from bread supplemented with malted barley flour (2-4%). DP = degree of polymerization.

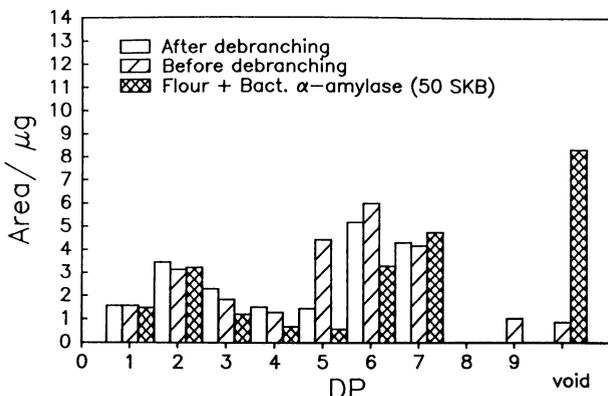


Fig. 7. Profile of dextrans extracted from bread crumb after precipitation of large dextrans with ethanol and resolubilizing with water. DP = degree of polymerization.

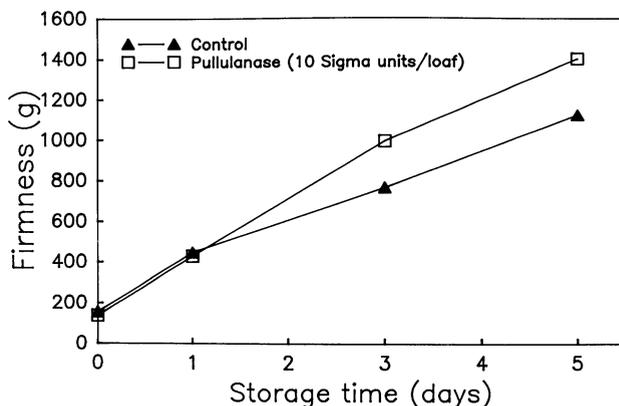


Fig. 8. Effect of pullulanase on bread firmness.

protein interface during storage and the fact that no new dextrins are produced during reheating. With no dextrins present, the bread firms at a rapid rate.

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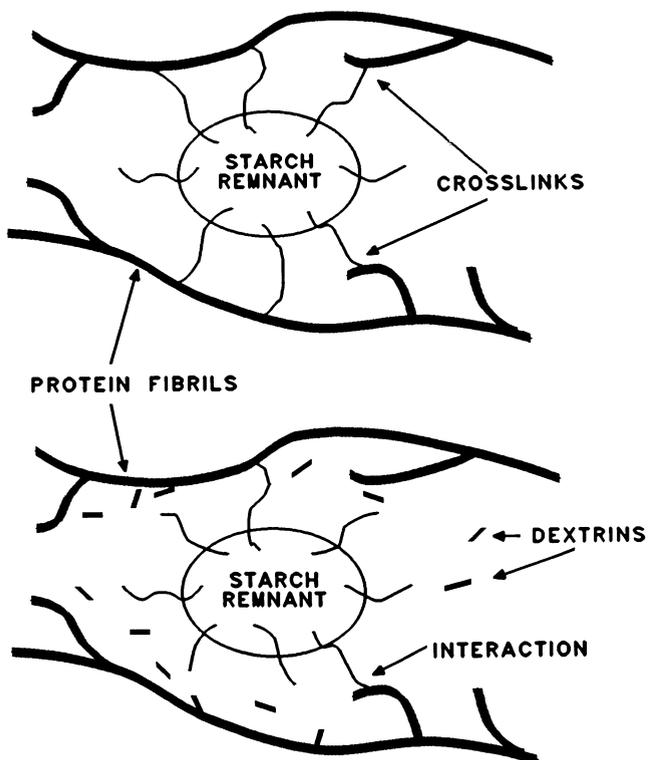


Fig. 9. Mechanism of bread firming and the antifirming role of dextrins.

link the protein fibrils, thus bread becomes firmer during storage.

The firming mechanism suggested herein accounts not only for the well-known antifirming effect of shortening and monoglycerides (restricting the swelling of starch) and the effect of certain amylases (production of certain dextrins), but also for a number of other factors. For example, the fact that bread firms more rapidly at a lower temperature is consistent with the effect of temperature on polymer interactions. The fact that bread can be refreshed with mild heating is explained by the heat-labile nature of hydrogen bonds and the effect of heat on polymer entanglements. Also of interest is the fact that reheated bread (as well as "brown-and-serve" bread) firms at a rapid rate. This can be explained by the diffusion of dextrins away from the starch-

[Received July 26, 1990. Accepted March 28, 1991.]