Bread Staling: X-Ray Diffraction Studies on Bread Supplemented with α-Amylases from Different Sources[1]

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ABSTRACT

The effects of barley malt, fungal α-amylase, and bacterial α-amylase on starch crystallization and organization in staling breads were studied by x-ray diffraction. Comparison of x-ray patterns of fresh and stored breads showed an order of decreasing degree of starch crystallinity as follows: bread with bacterial α-amylase, bread with cereal α-amylase, bread with fungal α-amylase, unsupplemented bread. Those results were in direct contradiction to bread firming data, indicating that starch crystallinity and bread firming are not synonymous. Starch crystalline organization also was affected by enzyme supplementation. Starches from breads containing bacteria enzyme supplements exhibited "V_h" and "A" structures, whereas the control had "V_h" and "B" structures. A possible role of starch crystal structure in bread firming was postulated.

Much evidence supports the hypothesis that bread staling largely results from changes in the starch component (Kim and D'Appolonia 1977, Maga 1975). Therefore, attempts have been made to retard staling by altering the physicochemical properties of starch.

Numerous reports have indicated that the addition of amylases to bread formulations reduces the firming of stored bread (Beck et al 1957, Becktel 1959, Miller et al 1953, Waldt and Maloney 1967). The reduction in bread firmness has been attributed to the enzymatic breakdown of starch (Miller et al 1953). Some authors have suggested that the dextrin fragments that are formed interfere with the crystallization of starch (Schultz et al 1952). However, on the basis of x-ray work done on breads made with gluten, cross-linked corn starch, and added bacterial α-amylase, Zobel and Senti (1959) offered another explanation. Their data showed that bacterial α-amylase increased, not decreased, starch crystallization. They postulated that bacterial α-amylase reduced bread firming by breaking links in the amorphous regions of the starch, thereby giving crystallites greater freedom of movement and a less rigid structure. However, other than providing an estimate of the changes in crystallinity during storage, they presented no x-ray patterns or interplanar spacings by which changes in starch crystalline organization could be evaluated.

The conditions for optimum activity of α-amylase are dependent on the enzyme source (Robyt and Whelan 1968). Breads supplemented with α-amylase from fungal and cereal sources maintain freshness longer than do unsupplemented controls (Miller et al 1953). Information on the effects of fungal and cereal α-amylase on the x-ray diffraction patterns of bread has not been published. Therefore, the objective of our study was to compare the effects of cereal, fungal, and bacterial α-amylases on starch crystallization and organization in staling breads. To accomplish this, x-ray diffraction patterns were made directly on fresh and stored breads and on the starch washed from those breads. By removing the other components of the bread crumb, we obtained x-ray patterns that more clearly revealed the crystalline organization of the starch.

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MATERIALS AND METHODS

Bread Preparation and Storage

Commercial hard red winter wheat flour (unmalted) containing 12.2% protein (14% moisture basis) and a falling number greater than 400 was used in this study. Bread was prepared by the sponge (70%) and dough (30%) process. The total formula consisted (in baker's percent) of 100% flour, 6% sucrose, 2% salt, 3% nonfat dry milk, 3% shortening, 2% yeast, 20 ppm $KBrO_3$, and an optimum amount of water. The sponge was fermented for 4 hr before the rest of the ingredients were added. The mixed dough was given 30 min floor time before molding, panning, and proofing to height. One-pound loaves were baked at 218°C for 25 min.

Doughs were made with and without enzyme supplementation. The enzyme sources and levels of supplementation in SKB units per 700 g of flour were barley malt, 400; fungal α-amylase (Amflex),[2] 400; and bacterial α-amylase (*Bacillus subtilis*),[3] 30.

After baking, the bread was allowed to cool on a rack 1 hr before being packed in polyethylene bags and stored at room temperature. Samples were taken daily for up to seven days for x-ray diffraction studies.

Measurement of Bread Firming

The Bloom gelometer was used to determine changes in bread softness during storage. Firming was measured as the grams of lead shot required to depress a plunger, 1 in. in diameter, a distance of 4 mm into a 1-in. thick slice of the bread crumb center. A greater number indicated greater firming. Three slices from each of the two loaves were tested.

X-Ray Diffraction

Starch was washed from fresh bread (30 min from the oven) and stored bread by immersing the crumb into an excess of distilled water, stirring gently for 30 min with a magnetic stirrer, passing the slurry through a 116-μm bolting cloth, and centrifuging the throughs at 2,000 rpm for 5 min. The supernatant was discarded; the pellet was resuspended in distilled water, centrifuged again, and then freeze-dried.

X-ray patterns of the starch (equilibrated to 96% rh) were taken with CuKα radiation on a Philips x-ray diffractometer. Operation was at 35 KV and 18 mA. Relative crystallinity of the starch was determined according to Herman's method as described by Nara et al (1978), in which the area of the crystalline fraction (a_c) is divided by the diffraction area for a 100% crystalline substance (A_c). The area of the crystalline fraction in the x-ray diffraction pattern of native (unheated) wheat starch was used as the value of A_c. X-ray patterns were designated according to the d-spacings and intensities given by Zobel (1964) and Zobel et al (1967). Broad peaks were divided into several peaks of equal width at half maximum intensity.

X-ray diffraction patterns of the actual bread were also made, utilizing CoKα radiation and a flat plate film cassette placed 2.5 cm from the sample. A bread slice of approximately 0.85 cm was cut, and the center section compressed to a thickness of 1 mm or less for placement in front of the exit port of the collimator. The specimen was covered on both sides with sheets of thin Saran to minimize moisture loss. Exposure time was set at 2.5 hr.

TABLE I
Effect of Various α-Amylase Supplementations on the Rate of Bread Firming[a] During Storage

Storage Time (hr)	Control	α-Amylase Supplementation: Barley Malt[b]	Fungal[b]	Bacterial[c]
2	46	43	45	32
16	100	74	79	46
24	107	80	88	48
48	130	110	113	50
72	176	136	141	52
96	221	154	158	54
120	230	182	190	55
168	253	222	234	58

[a] Grams of lead shot required to depress plunger, 1-in. in diameter, into the crumb a distance of 4 mm.
[b] 400 SKB units per 700 g of flour.
[c] 30 SKB units per 700 g of flour.

RESULTS

Bread Firming During Storage

Data on the firming of bread crumb during storage as affected by α-amylase supplementation are shown in Table I. Unsupplemented breads and breads containing barley malt or fungal enzyme supplements showed essentially the same initial softness in the fresh (2 hr) product. However, fresh breads containing bacterial α-amylase were considerably softer.

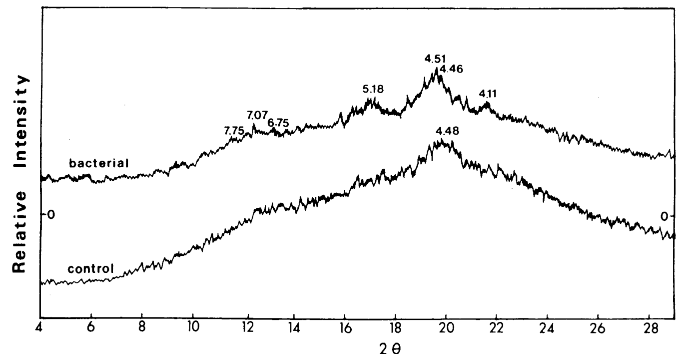

Fig. 1. X-ray diffraction (CuKα) patterns of starch extracted from fresh breads, unsupplemented and supplemented with bacterial α-amylase. Numbers above peaks indicate d-spacings in Å.

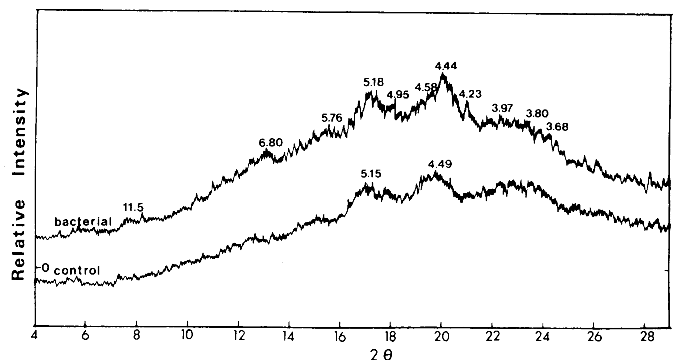

Fig. 2. X-ray diffraction (CuKα) patterns of starch extracted from one-day-old breads, unsupplemented and supplemented with bacterial α-amylase. Numbers above peaks indicate d-spacings in Å.

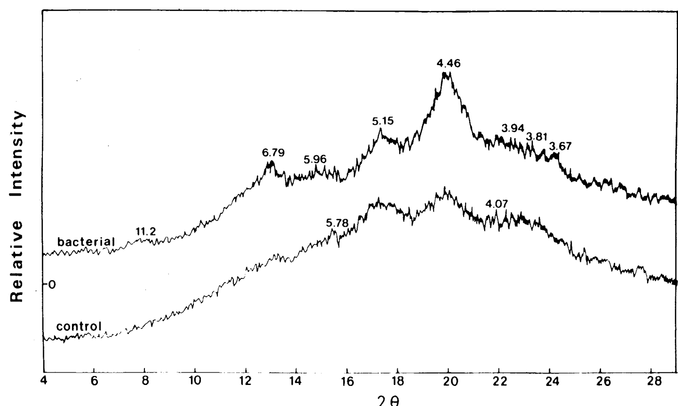

Fig. 3. X-ray diffraction (CuKα) patterns of starch extracted from seven-day-old breads, unsupplemented and supplemented with bacterial α-amylase. Numbers above peaks indicate d-spacings in Å.

All breads showed increased firming during storage, with the greatest increase occurring between 2 and 16 hr. Furthermore, unsupplemented breads showed the fastest rate of firming; breads supplemented with bacterial α-amylase showed the slowest rate. Breads containing barley malt or fungal enzyme supplements exhibited intermediate rates of bread firming during storage. Those data agree with the early findings of Miller at al (1953) and, in keeping with current theories on bread staling (Kim and D'Appolonia 1977), suggest that the starch in unsupplemented breads crystallizes at a faster rate than does the starch in enzyme-supplemented breads.

X-Ray Diffraction

Because x-ray diffraction studies on breads and on starch washed from breads gave essentially the same results, only the patterns of the isolated starch will be discussed.

X-ray diffraction patterns of starch washed from unsupplemented breads and breads supplemented with bacterial α-amylase are shown in Figs. 1–3. The d-spacings of the peaks that were identified as well as the comparative intensities of those peaks are given in Table II. Starches from both types of bread (Fig. 1) have V-hydrate (V_h) patterns, as indicated by strong d-spacings at 4.46 or 4.48 Å. However, starch from fresh bread supplemented with bacterial α-amylase is more crystalline than that of the control, as shown by the larger number of discernible peaks and by the relative crystallinity determinations (Table III). That pattern also has several peaks whose d-spacings (7.75 Å, 5.18 Å) and relative intensities suggest that they fit into the type A starch structure.

Zobel and Senti (1959) found that fresh breads containing cross-linked corn starch and bacterial α-amylase had a more intense line 5 (5.16 Å) than did the control. Because of the intensity of that line, they suggested that a greater proportion of the starch in fresh breads was in the B structure. However, line 5 can also be a part of the A and C structures. Without some indication of the d-spacings of the other lines in the pattern and a designation of the comparative intensities of the various lines, the assignment of a crystalline structure could be ambiguous.

TABLE II
Effect of Various α-Amylase Supplementations in Breads on d-Spacings (Å) of Starch Washed from Fresh and Stored Bread Crumb[a]

Storage Time (days)	Control	α-Amylase Supplementation: Bacterial	Barley Malt	Fungal
0[b]	6.75 vw	7.75 vw	6.80 w	7.53 vw
	4.48 s	7.13 w	5.15 w	6.80 w
		6.75 vw	4.45 s	6.41 vw
		5.18 w	4.26 vw	4.44 s
		4.51 s	3.73 vw	4.13 vw
		4.46 s	3.60 vw	
		4.34 w		
		4.11 vw		
1	5.82 vw	11.5 vw	6.75 vw	7.22 vw
	5.15 m	7.78 vw	5.75 vw	6.75 vw
	4.98 w	6.80 w	5.12 m	5.81 w
	4.49 s	5.76 w	4.75 m	5.18 w
	3.97 m	5.18 s	4.44 s	5.12 m
	3.82 m	4.95 w	4.07 w	4.79 w
	3.68 m	4.58 m	3.93 w	4.43 s
		4.44 s	3.79 w	4.09 vw
		4.23 w		3.83 w
		3.97 w		3.72 w
		3.80 w		3.67 w
		3.68 w		
7	7.01 vw	11.2 vw	7.69 vw	5.12 m
	6.71 vw	6.79 m	7.07 vw	4.82 m
	5.78 vw	6.55 w	5.81 vw	4.44 s
	5.15 s	5.96 w	5.12 m	4.17 w
	4.97 m	5.15 m	4.92 w	4.04 w
	4.45 s	4.46 vs	4.48 s	3.89 m
	3.96 m	4.04 vw	4.37 s	3.80 m
	3.83 m	3.94 vw	3.94 vw	
	3.75 m	3.81 vw	3.86 w	
		3.67 vw	3.70 w	

[a] Intensity scale: vs = very strong, s = strong, m = medium, w = weak, vw = very weak.
[b] 30 min out of the oven.

After one day of storage, starch crystallinity in control and enzyme-supplemented breads increased, especially when breads were supplemented with bacterial α-amylase. The increased intensities of the x-ray patterns are clearly shown in Fig. 2. The d-spacings and intensities of the individual peaks (Table II) suggest that the starch in one-day-old bread supplemented with bacterial α-amylase has a strong V_h pattern superimposed on a weak A structure. Conversely, the starch in the one-day control appears to have a mixture of the V_h and B structures. The strong 15.8 Å spacing typical of the B structure was only a very weak peak in our patterns. This may have been due to small fluctuations in the moisture content of the samples. Zobel and Senti (1959) have noted that the 15.8 Å spacing is very sensitive to small changes in moisture content.

An even more dramatic increase in starch crystallinity occurred when breads supplemented with bacterial α-amylase were stored for seven days (Fig. 3). The relative crystallinity increased from 0.65 to 0.91 (Table III). The CuKα x-ray diffraction of the starch from breads stored one day and seven days gave identical and superimposable patterns except in the 2θ angular region from 12.5–24° (Fig. 4). The 6.8 and 4.4 Å peaks increased noticeably during the staling period, whereas the entire diffraction regions from 6.5 to 4.8 Å as well as from 3.9 to 3.6 Å decreased significantly. An apparent decrease in the A starch structure with concomitant increase in the V_h starch structure of the stored bread occurred.

The x-ray crystal structure of the control starch changed very little during one to seven days of storage (Fig. 3), but the degree of crystallinity increased from 0.51 to 0.62.

Starch crystalline organization and structure in staling breads containing barley malt or fungal α-amylase were very similar, so only one figure in presented (Fig. 5). Starch from the fresh and stored breads was more crystalline than was starch from the control but less crystalline than was starch from breads supplemented with bacterial α-amylase (Table III). In general, the crystalline structure of the barley malt and fungal starch consisted of a V_h structure in the fresh samples and a mixture of V_h and A and B structures in stored breads.

DISCUSSION

X-ray diffraction patterns of breads supplemented with different sources of α-amylase showed that the enzyme-supplemented breads exhibited greater crystallinity, initially and during storage, than did the unsupplemented control. Comparison of the bread treatments showed an order of decreasing degree of starch crystallinity as follows: bread with bacterial α-amylase, bread with cereal α-amylase, bread with fungal α-amylase, and bread with no supplementation (Table III). The degree of crystallinity paralleled the heat stability of the enzymes; bacterial α-amylase has the highest thermostability, followed by cereal α-amylase, whereas fungal α-amylase is heat labile (Kulp 1975). On the other hand, breads supplemented with bacterial α-amylase

TABLE III
Changes in the Relative Crystallinity[a] of Starch During Storage of Control and Enzyme-Supplemented Breads

Treatment	Storage Time, days: 0	1	7
Control	0.32	0.51	0.62
Bacterial α-amylase	0.48	0.65	0.91
Barley malt	0.42	0.59	0.70
Fungal α-amylase	0.39	0.55	0.65

[a] Relative crystallinity = a_c/A_c, where a_c is the area of the crystalline fraction and A_c is the crystalline diffraction area for intact (unheated) wheat starch.

produced the softest bread crumb (Table I). Therefore, those data indicate that increases in starch crystallinity are not synonymous with bread firming.

An explanation for the increase in the rate and degree of starch crystallization in breads supplemented with bacterial α-amylase has been suggested by Zobel and Senti (1959) and is based on well-established concepts in polymer chemistry (Lenz 1967). Undegraded starch polymers (amylose and amylopectin molecules in unsupplemented breads) are bulky and cumbersome, so more time is required for the segments to rotate into equilibrium lattice positions. Consequently, the overall rate of crystallization is slow. On the other hand, enzymatic degradation of the starch polymers in amylase-supplemented breads produces lower molecular weight species. Crystallization is hastened because these smaller units have more freedom of movement and can more easily arrange themselves into lattice position. In addition, new crystallites can grow out from nuclei that are potentially present in the amorphous structure wherever chain segments happen to be parallel in lattice positions. The net result would be an increase in the degree of crystallinity of the system.

The mechanism by which cereal and fungal α-amylase affect starch crystallinity is uncertain. However, based on data reported in the literature, we suggest that the mechanism postulated for bacterial α-amylase also operates when amylases of fungal and cereal origins are used. The inactivation temperature of the various enzymes in the bread environment is the limiting factor. At 70°C, 92 and 52% residual α-amylase activity remains when suspensions of flour and cereal or fungal α-amylase, respectively, are heated in the amylograph (Miller et al 1953). Walden (1955) showed that the center of a 1-lb loaf of bread takes about 14 min to reach 70°C at a baking temperature of 230°C, and that at 70°C more than 80% of the cereal α-amylase activity remains when starch-amylase systems are subjected to the time-temperature conditions that exist in a loaf of bread. Therefore, in the first 14 min of baking, the conditions in the center of a loaf of bread are probably conducive to the activity of α-amylase from bacterial, fungal, and cereal sources. The higher inactivation temperature of bacterial α-amylase allows for greater conversion of starch to lower molecular weight species and accounts for its greater effect on starch crystallinity.

Although an increase in starch crystallinity in the unsupplemented breads during storage paralleled increased firming (Tables I and III), crystallinity alone cannot fully explain bread firming. Changes in starch crystalline organization also may be an important factor. All starches showed the V_h structure, initially and throughout storage. During storage, mixtures of the V_h and A and B structures were observed in starches from breads that were supplemented with barley malt or fungal amylases. However, starch from the control bread developed a combination of V_h and B structures, whereas starch from bacterial α-amylase supplemented breads exhibited V_h and A structures. In the breads supplemented with bacterial α-amylase, the A starch structure appears to decrease and the V_h starch structure to increase with storage. The moisture in the gluten is possibly absorbed by the A starch, increasing the diameter of the starch helices and shortening the coil length. The V_h starch helix is roughly 12% longer than that of the A starch. Although the coil length (c-axis) is shorter, the 12% increase results from the larger helical diameter, which could be accounted for by the addition of 8–12 water molecules for the 12 glucose molecules in the two starch unit cells.

The significance of the differences in x-ray crystal patterns with respect to bread firming could be their effect on the water content of the unit cell. The B structure can hold more water molecules in the unit cell than the A structure can, 36 versus 8 molecules, respectively (Sarko and Wu 1978). If, as recent evidence suggests (Breaden and Wilhoft 1971; Wilhoft 1971a, 1971b), moisture is transferred from the gluten to the starch phase during aging, with a resultant volume decrease in the gluten, then more water molecules could be transferred from the gluten to the B starch of the control than to the A starch in enzyme-supplemented breads. The gluten in control breads would therefore have less moisture content than its enzyme-supplemented counterparts would, which could be responsible, in part, for its increased rate of crumb firming. Wilhoft (1971b) has suggested that the ratio of starch to gluten (6:1) in bread crumb ensures that moisture transfer to the starch would result in the firming of the continuous gluten phase.

CONCLUSION

Our data show that although starch crystallizes in breads during storage, starch crystallization and bread firming are not synonymous. Data on changes in starch crystalline structure, particularly on the unit cell's water content, suggest that cell water content may be important in the transfer of moisture from the gluten to the starch during storage. We propose that if a strong V_h (possibly mixed with a little A) is formed in the starch during baking, little space would be available in that crystal structure for water transfers from the gluten. If that is so, flexibility of the gluten phase would be preserved, which would reduce bread firming during storage.

The importance of the V_h structure in the bread softening effect of amylases could also explain the bread softening action of other additives. For example starch in breads containing sodium stearoyl-2-lactylate exhibits a strong V_h pattern, as a result of the complexing of fatty substances with starch molecules, whereas breads containing none of this additive show a weak V_h pattern.[4] The V complex may prevent the release of starch solubles from the

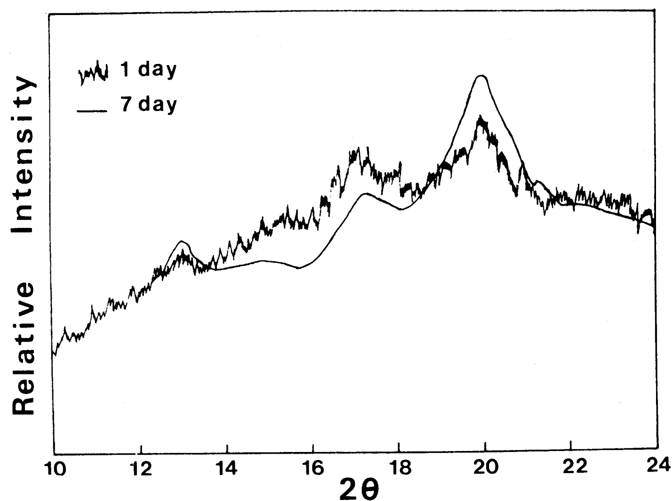

Fig. 4. X-ray diffraction (CuKα) patterns of starch extracted from breads supplemented with bacterial α-amylase, one and seven days old. The line is the averaged intensity for the seven-day starch shown in Fig. 3.

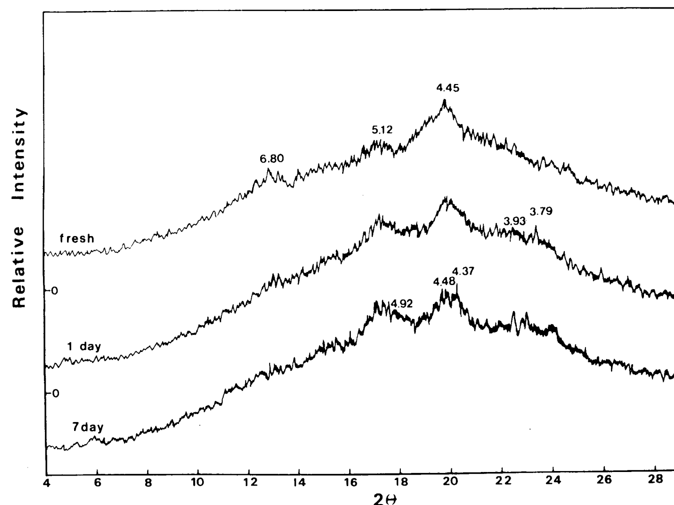

Fig. 5. X-ray diffraction (CuKα) patterns of starch extracted from fresh and stored breads supplemented with barley malt or fungal α-amylase. Numbers above peaks indicate d-spacings in Å.

granule during baking and thus retard bread firming. On the other hand, surfactants, by forming a strong V_h structure with starch, may lessen the redistribution of moisture from gluten to starch and thus prevent contraction and subsequent firming of the gluten phase. More work is needed to define the separate roles of starch crystallinity and starch structure in the staling process.

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