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Solid-State ^{13}C CP/MAS NMR Studies on Aging of Starch in White Bread

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The effects of storage methods and glycerol on the aging of breadcrumbs were studied using solid-state ^{13}C CP/MAS NMR. After baking, a shift in C_1 peaks from triplet (A-type) to singlet (V-type) was observed. Addition of glycerol reduced the carbon peak intensities of fresh and aged breads, which correlated well with the DSC amylopectin "melting" enthalpy ($r^2 = 0.91$). Upon storage of bread with crust in hermetically sealed containers (when moisture migrated from the crumbs to the crust), the ^{13}C CP/MAS NMR peak intensity increased more rapidly during aging than when the bread was stored without crust. Although addition of glycerol retarded the starch retrogradation, as observed by ^{13}C CP/MAS NMR and DSC, it accelerated the firming rate. Therefore, bread firming in this case was controlled not only by starch retrogradation but also by other events (such as local dehydration of the matrix or gluten network stiffening).

KEYWORDS: White bread; ^{13}C CP/MAS NMR; starch retrogradation; staling; firming

INTRODUCTION

Starch retrogradation has been long believed to be the major cause of staling of bread, leading to increased firmness (1–3). Many studies have been done to monitor amylopectin recrystallization as an indicator of the degree of retrogradation (4–13). Other factors may also play a role, for instance, cross-link formation between the continuous gluten phase and the discontinuous phase of swollen starch granules and leached amylose (14, 15). Moisture migration has been suggested to be a contributing factor (4, 8, 16–19).

In shelf-stable breads, glycerol is sometimes added to reduce bread water activity and to soften the bread, but the effect of glycerol on the staling mechanism is not well understood. Meal Ready-to-Eat (MRE) bread containing glycerol has been characterized using differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA), and when it was stored in a hermetically sealed pouch, only a small firming increase was reported for up to 3 years (9). The potential benefit from addition of glycerol, however, can diminish if bread is stored with the crust intact, in that moisture loss from crumb to crust strongly affects the texture (20). Amylopectin recrystallization observed from MRE bread evidently did not lead to a significant adverse effect on texture (21). A thermomechanical study on fresh and aged breads has suggested that local dehydration of the amorphous network could be observed upon aging of bread,

elevating the thermal transition range to higher temperatures (12).

Other properties may also contribute to crumb firming. The association of gelatinized starch granules with gluten can play an important role in bread firming (14) through hydrogen-bonding (and other) interactions (22). Starch has been an important factor, as evidenced in studies of gluten-free and α -amylase-treated starch bread (3).

Since the effect of starch on bread firming can result from crystalline and rigid amorphous starch chains, this study applied solid-state ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy to investigate both amorphous and crystalline starch (23–27). This technique has been applied previously to monitor the development of rigid components in starch (3, 26, 28). Upon gelatinization of the sample, a ^{13}C CP/MAS NMR spectrum exhibits a significant loss in signal intensity (26), as more mobile, liquid-like starch components are formed. During storage, some of the mobile, amorphous starch became more rigid and some recrystallized, resulting in an increased signal intensity (3, 26, 29). But retrograded starch may not necessarily be crystalline (3, 26). Molecular ordering of linear polyglucose chains can occur in a short range (not appearing as crystalline order as determined by X-ray diffraction). Alignment of the double helices in a long-range order eventually occurs, leading to development of a corresponding crystalline pattern that can be observed by X-ray diffraction. ^{13}C CP/MAS NMR spectroscopy has been applied to observe the development of glassy and rigid starch components upon cooling, shown as a higher ^{13}C CP/MAS NMR intensity (29). Additionally, the development of rigid amorphous and crystalline starch components during aging of bread has been measured using this technique (3, 26).

Table 1. Formulation of White Bread (in Grams)

component	control	2.6% glycerol (g of glycerol/ 100 g of wheat flour)	8.8% glycerol (g of glycerol/ 100 g of wheat flour)
wheat flour	200.00	200.00	200.00
water	120.00	128.90	142.70
shortening	10.00	10.00	10.00
sugar	9.40	9.40	9.40
nonfat dry milk	6.00	6.00	6.00
active dry yeast	5.40	5.40	5.40
salt	4.50	4.50	4.50
calcium propionate	0.96	0.96	0.96
potassium sorbate	0.48	0.48	0.48
glycerol		5.28	17.60
moisture content after baking (g of water/ 100 g of bread)	39.0 ± 0.1	38.5 ± 0.5	39.5 ± 0.5

DSC, DMA, and mechanical techniques are limited to long-range (structural) applications. For short-range molecular studies, it is desirable to apply methods such as ^{13}C CP/MAS NMR. In this study, the molecular rigidity changes of starch in white bread containing glycerol (stored with and without crust) were investigated. The purpose was to determine the effect of glycerol in situations with and without water migration on starch molecular rigidity, as observed by solid-state ^{13}C CP/MAS NMR. Comparison was made with amylopectin “melting” or “staling” endotherms analyzed by DSC (20) and firmness data (30) from earlier reports.

MATERIALS AND METHODS

Materials. Wheat flour [unbleached, all-purpose, 10% protein, 73.3% carbohydrate (starch), and 16.7% water], shortening, sugar, nonfat dry milk, active dry yeast, and salt were purchased at a local grocery store. Additives used were potassium sorbate (Sigma Chemical Co., St. Louis, MO), calcium propionate (Pfizer Inc., New York, NY), and glycerol (Fisher Scientific, Philadelphia, PA). Polyethylene, used as an internal NMR standard, was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Bread. The formulation of the standard white bread is shown in **Table 1**. Standard white bread containing 0%, 2.6%, and 8.8% glycerol (wheat flour basis) was made using an automatic breadmaking machine (Bread Bakery model SD-BT51P, Panasonic, Secaucus, NJ) and a general baking method. The mixing time was 20 min, the resting time was 5 min, and the kneading period was 5 min. The dough was allowed to rise for 160 min, and then it was baked at 160 °C for 50 min. This bread was cooled at room temperature for 1 h before being packed. After baking, two storage methods were used: (1) each loaf of bread was packed with the crust intact or (2) the crust was removed by cutting a loaf into 15-mm × 15-mm × 50-mm (height × width × length) shapes. Each sample (intact breads and crumbs) was hermetically sealed in a trilaminated pouch (Cadillac Products Inc., Dallas, TX) to prevent moisture loss to atmosphere and then stored at 25 °C.

Moisture Content. Moisture content was determined using a vacuum oven drying method at 70 °C and 29 in. Hg overnight (AOAC method 925.09) and calculating from the weight change.

X-ray Diffraction Analysis. Breadcrumb samples were dehydrated using ethyl alcohol (Fisher Scientific, Philadelphia, PA) for 2 days and then gently ground using a mortar. The dried sample was packed in a capillary tube (1.98 mm internal diameter, 0.01 mm walls, 80 mm length; Charles Supper Co. Inc., Natick, MA). X-ray diffraction analysis was done using a Siemens D 500 diffractometer (Siemens, Munich, Germany) operating in a transmission mode with a Ni filter and Cu K α radiation. The X-ray source had a wavelength of 0.15418 nm, and the diffraction pattern was recorded at angles (2θ) of 2–40°.

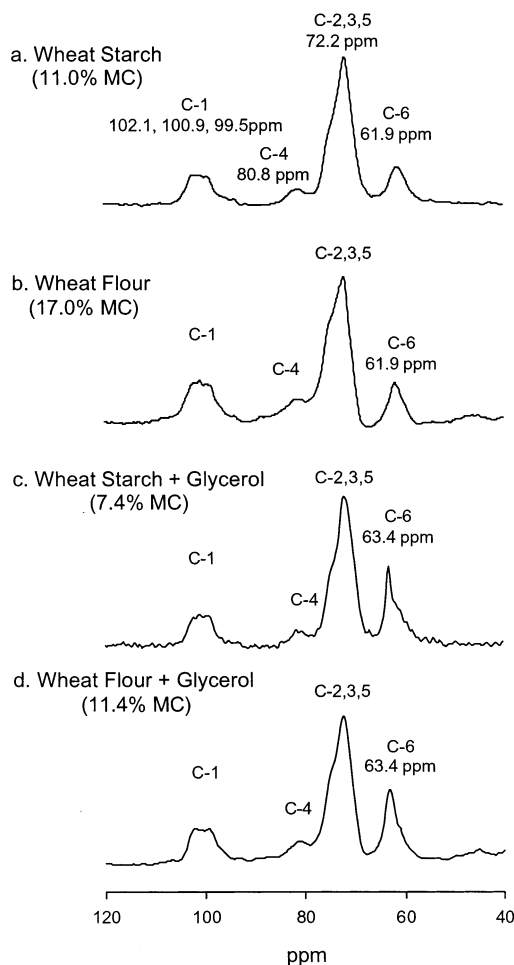


Figure 1. ^{13}C CP/MAS NMR spectra of (a) wheat starch, (b) wheat flour, (c) wheat starch and glycerol mixture (2:1), and (d) wheat flour and glycerol mixture (2:1). %MC is percent water total weight basis.

^{13}C CP/MAS NMR Analysis. The ^{13}C CP/MAS NMR spectra were recorded at 75 MHz using a Bruker MSL 300 NMR spectrometer (Great Britain Bruker Spectrospin Ltd., Coventry, UK) operating at room temperature. Each breadcrumb sample (150 ± 2 mg) was taken from the center of a loaf of bread using forceps and packed into a 7-mm ceramic rotor with 10 mg of polyethylene added (as internal reference) and spun at 2 kHz. The ^{13}C CP/MAS NMR spectra were obtained with a cross-polarized (CP) pulse sequence, applying a contact time of 2 ms and a repetition time of 3 s, which had been determined previously for dough (31). The accumulation of at least 10 000 scans was done to obtain a satisfactory signal-to-noise ratio. The relative peak intensity of each carbon group was calculated from the ratio between the peak area of each carbon group and the peak area of polyethylene.

Statistics. In each experiment, three loaves of bread were baked in three different bread machines at the same time. All moisture content determination gave errors within 2%, and only the average values are shown here. ^{13}C CP/MAS NMR peak intensities are presented as average and standard deviation (0–15%). Statistical significance was tested by the Duncan test using SAS software (version 6.12, SAS Institute, Inc., Cary, NC) at the 95% confidence level. Regression analysis (rate constant calculation and linear regression) was performed using Sigma Plot software (version 4.01, Jandel Scientific, San Rafael, CA) at the 95% confidence level.

RESULT AND DISCUSSION

^{13}C CP/MAS NMR Spectra of Wheat Starch and Flour. **Figure 1** shows a typical ^{13}C CP/MAS NMR spectrum of wheat starch and wheat flour. Carbon chemical shifts for starch have been identified: 96–102 ppm for C₁, 70–73 ppm for C₂, C₃,

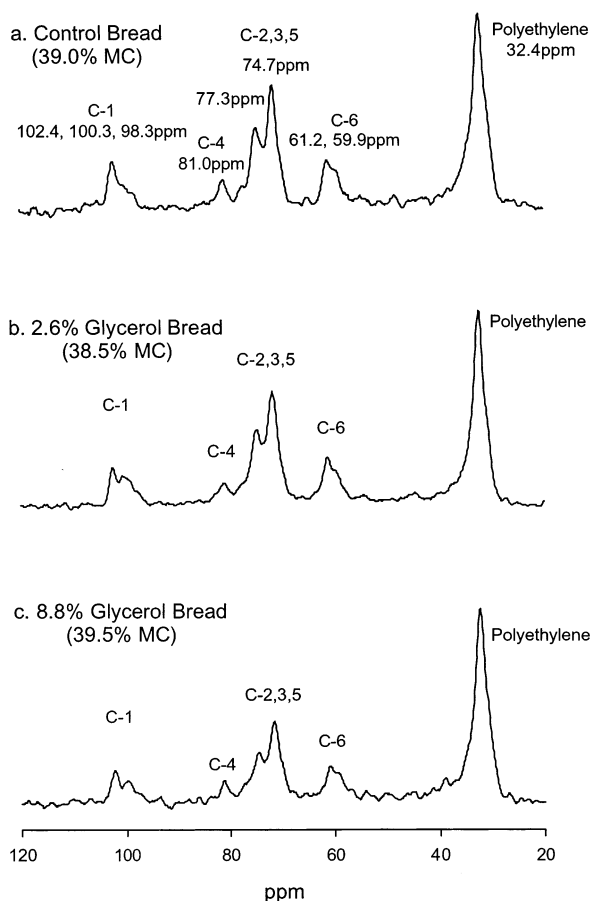


Figure 2. ^{13}C CP/MAS NMR spectra of fresh breadcrumbs with 0%, 2.6%, and 8.8% glycerol (grams of glycerol per 100 g of wheat flour). %MC is percent water total weight basis.

and C_5 , 77–83 ppm for C_4 , and 59–62 ppm for C_6 (27). No significant gluten peak was observed (therefore not shown in **Figure 1**). The C_1 position of the glucose units exhibits characteristic chemical shift patterns that can reveal the nature of crystallinity in starch (23, 26, 27). For an A-type crystal, which has three nonidentical sugar residues, the C_1 peak exhibits a triplet pattern at ~ 102 , 101, and 100 ppm whereas, for a B-type crystal, which has two nonidentical sugar residues, the C_1 becomes a doublet each at ~ 101 and 100 ppm (32). Our results for starch and flour samples showed a triplet pattern indicating an A-type crystal (**Figure 1**).

The wheat flour used in this experiment contained about 88% (dry basis) of starch and 12% (dry basis) of protein. The ^{13}C CP/MAS NMR spectra of wheat flour show that major signals are due to sugar carbons in the starch component of the flours (33, 34). Line assignments in the spectra are protein side-chain aliphatic carbon (20–35 ppm), starch (60–105 ppm), protein side-chain aromatic carbon (~ 130 ppm), and protein main-chain peptide carbonyl carbon (~ 175 ppm) (34). In our experiment, unfortunately, we could not observe the signal from the protein clearly.

When glycerol was added to wheat starch and flour, the spectra showed little change (**Figure 1**). This peak is not likely to be due to glycerol since typical glycerol carbon NMR peaks are at 66.9 and at 77.4 ppm (35). However, it is possible that glycerol affects the dynamics of the molecular groups around the C_6 region. According to the cluster model of amylopectin in starch granules (36), the α -1–6 linkage part is mostly amorphous. Glycerol might therefore affect some of the C_6 carbons in the amorphous chains. Moistened starch has been

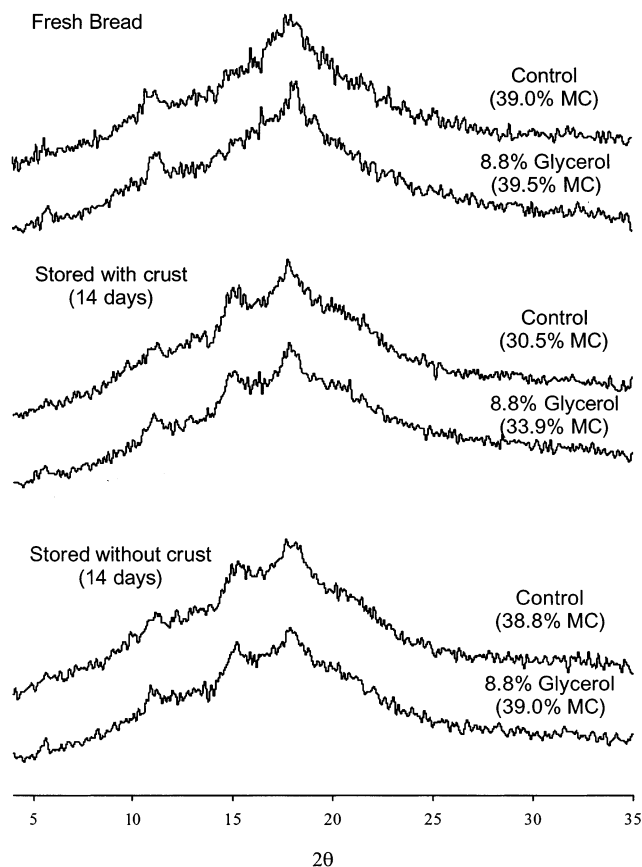


Figure 3. X-ray diffraction patterns of breadcrumbs, comparing fresh and aged samples containing 0% and 8.8% glycerol (flour basis). %MC is percent water total weight basis.

shown to have a narrower ^{13}C NMR line width than dry starch (32, 37–39). The presence of glycerol could give an additional line narrowing effect, since it can lead to a further increase in mobility of the starch chains. Such plasticizing effects can promote ordered structure formation (32, 37, 39), aiding in the formation of the helical structure (38).

^{13}C CP/MAS NMR Spectra of Bread. **Figure 2** shows ^{13}C CP/MAS NMR spectra for fresh breadcrumbs containing 0%, 2.6%, and 8.8% glycerol (flour basis). The peak at 32.4 ppm is the resonance of the polyethylene internal standard. These spectra were similar to those reported in starch gel (26, 28, 29) and starch (reconstituted) bread (3). In the control sample, the C_1 resonance shows a strong peak at 102.4 ppm, characteristic of a V pattern (37), with smaller peaks at 100.3 and 98.3 ppm. There may be another smaller peak hidden within the first peak (102.4 ppm), which means that A-type might also be present. A presence of A-type starch is possible if the original starch is partially melted in the baking process. However, X-ray diffraction patterns of fresh bread (**Figure 3**) showed a weak crystalline pattern and are not conclusive. In aged bread, the X-ray diffraction pattern showed a trace of a B-type crystal rather than an A-type crystal (as noted from the appearance of a peak around $2\theta = 15^\circ$, **Figure 3**). It has been reported that in some wheat bread, amylopectin recrystallized into a B-type (40). What we observed here is a weak crystalline pattern. Most C_1 resonances showed one sharper peak at 102.4 ppm with a shoulder (**Figure 2**) which upon aging became stronger (**Figure 4**). ^{13}C CP/MAS can detect a rigid amorphous chain and B-chains as a duplex. Upon storage, the observed development of a duplex with a broad shoulder suggested that there could be a development of a B-type configuration with some degree of rigid amorphous

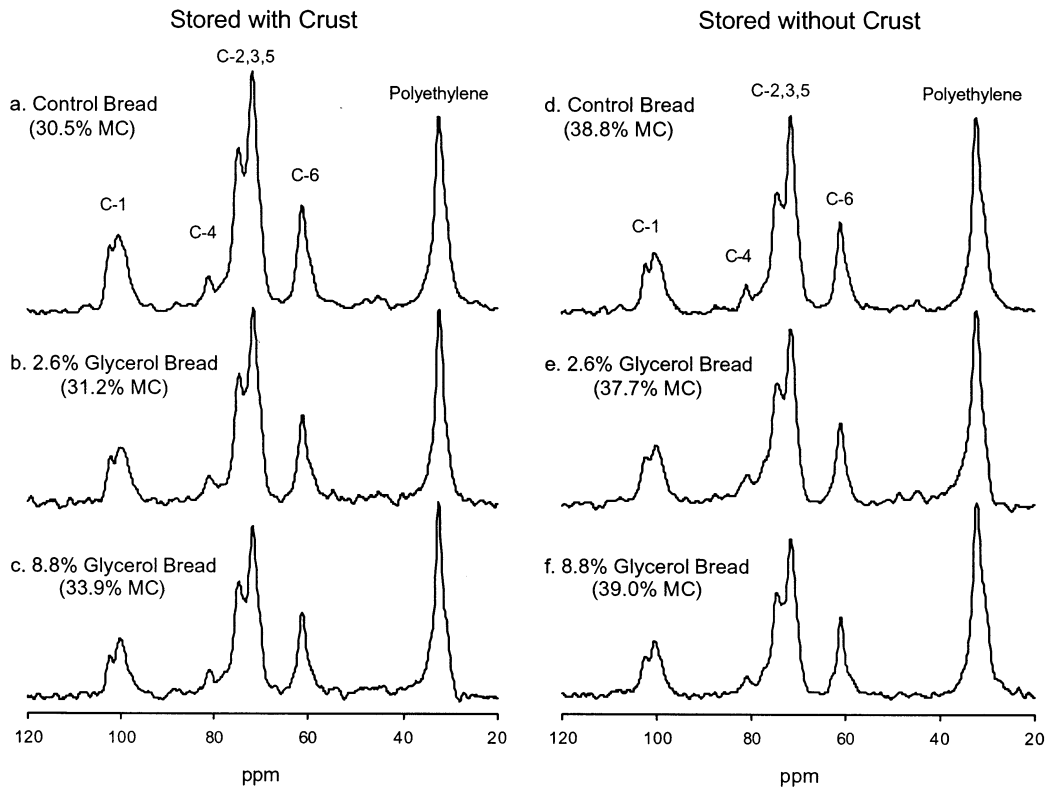


Figure 4. ^{13}C CP/MAS NMR spectra of aged (14 days) breadcrumbs with 0%, 2.6%, and 8.8% glycerol (grams of glycerol per 100 g of wheat flour). The peak at 32.4 ppm is due to polyethylene, which is used as an internal intensity reference. %MC is percent water total weight basis.

chains. The fact that there was only a slight (at best) increase in the X-ray diffraction peak seems to suggest that these B-type double helices did not align in a long-range crystalline order.

Addition of glycerol up to 8.8% (flour basis) leads to spectra similar to those of the fresh control but with lower signal intensity (**Figure 4**), but when the peak area was calculated in terms of relative intensity (with respect to the internal standard (polyethylene)), addition of glycerol reduced the relative signal intensity of C_1 and C_6 (**Figure 5**; $\text{C}_{2,3,4,5}$ represents unresolved relative peak areas between 68 and 85 ppm). We will focus on the C_1 and C_6 data. Because of the weight of added glycerol, some intensity decrease was expected with glycerol concentration (dotted lines, **Figure 5**). The observed intensity, however, decreased with glycerol content far more than expected (solid line, **Figure 5**). All breadcrumbs being compared contained practically the same amount of water ($39.0 \pm 0.5\%$). The difference observed here is strong evidence that glycerol lowers crystalline and other rigid components in the starch fraction in fresh breadcrumbs. This might be a combined effect along with amylose crystallization inhibition. Additionally, doughs containing glycerol also were varied in terms of the amount of water added in order to obtain bread with the same final moisture content after baking. Adding the same amount of water with varying glycerol contents in the dough stage led to a decrease of the moisture content in fresh breadcrumbs (after baking) with increasing glycerol contents (data not shown). The aim of this experiment was to compare the role of glycerol when the same amount of a plasticizer other than glycerol (i.e., water) was present in the breadcrumbs.

The effect of glycerol is complex. Making breads with added glycerol with the same dough moisture content can lead to final breads with different moisture contents (and also other properties, such as fermentation, bread porosity, degree of gelatinization, and microstructure). To prepare breads with various

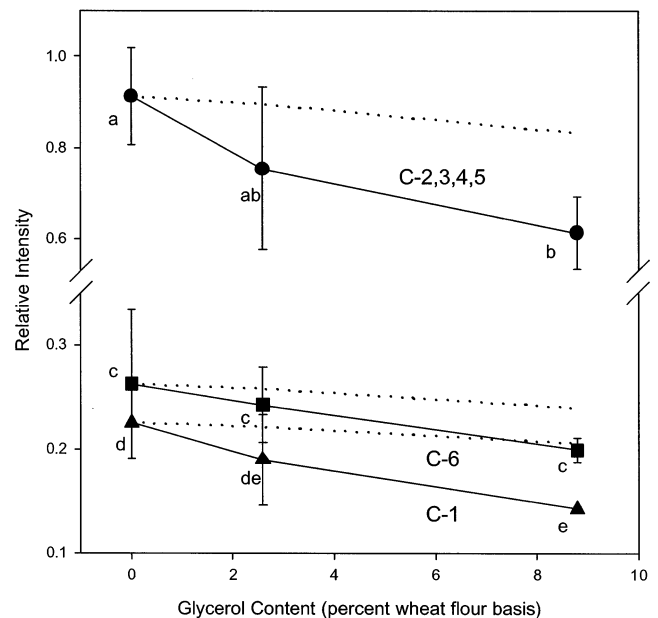


Figure 5. Relative ^{13}C CP/MAS NMR spectral intensity for starch in fresh breadcrumbs at various glycerol contents (moisture content $39.0 \pm 0.5\%$, wet basis). Solid lines are observed experimental values, and dotted lines are no-effect baseline (calculated by assuming no change in starch and corrected for dilution effect of glycerol). Points with different letters within the same line are significantly different at $p \leq 0.05$.

amounts of added glycerol but the same moisture content after baking, we modified the amounts of water added to the dough (**Table 1**). In an earlier investigation, we also did experiments with breads made from dough containing the same moisture content (but different glycerol contents). Water migration, DSC endotherms, and X-ray diffraction results were obtained and

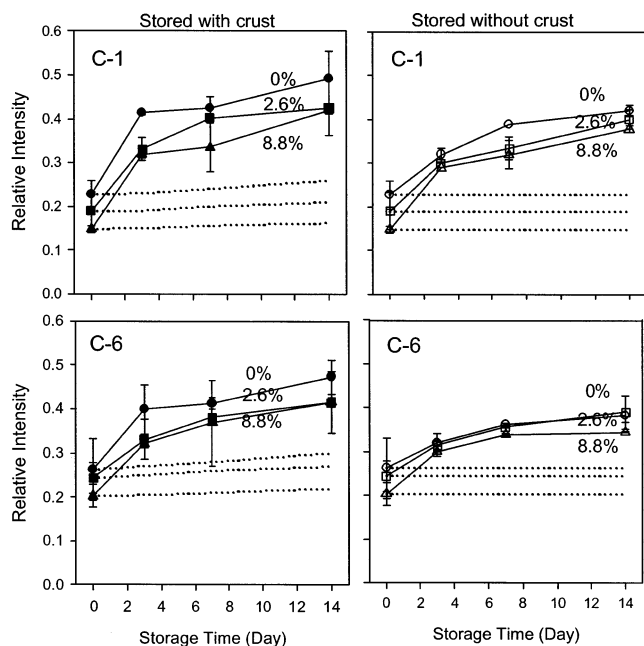


Figure 6. Change of C_1 and C_6 peak intensities of starch in breadcrumbs during storage at 25 °C. Solid lines and symbols are experimental data, and dotted lines are no-change baseline (assuming no change in starch except for some moisture loss).

reconfirmed similar retrogradation in terms of both DSC, X-ray diffraction crystallinity, and water migration from crumb to crust (20).

The change in ^{13}C CP/MAS NMR peak intensity has been related to a change in molecular mobility, with higher ^{13}C CP/MAS NMR peak intensities being due to a decrease in segmental mobility resulting in more efficient cross-polarization (41). It has been reported that ^{13}C CP/MAS NMR peak intensity of both fresh and aged starch gels increased with decreasing temperature from 10 to -10 °C, similar to the result found for the rubbery-to-glassy transition in starch gel (29). When rubbery, amorphous domains become more rigid through the rubbery-to-glassy transition, an increase in ^{13}C CP/MAS NMR peak intensity results. This is because the process in rigid solids (glassy) is more effective than that in mobile rubber solids (28, 29, 41). The higher peak intensity in aged starch gel is most likely due to the greater degree of molecular rigidity or immobilization of the aged sample, which could be caused by various factors including increased crystallinity, rubbery–glassy changes in the amorphous domains, and water redistribution (29). However, X-ray diffraction data did not show a very significant increase in crystallinity, indicating that the rigidity observed by NMR was only from the short-range molecular order.

When aged, all peak intensities of all bread samples increased significantly during storage. All spectra showed patterns similar to those shown in **Figure 4** for all breads. Breads that contained added glycerol (both concentrations) but different amounts of water in the dough stage and were stored without crust (hence no moisture loss) showed C_1 resonances strongly resembling those of B-type starch (clear doublet, **Figure 4**). The relative intensities for C_1 and C_6 resonances were analyzed (**Figure 6**). The peak intensity increased significantly over storage time in all breadcrumb samples. Comparing the data for bread with and without crust, the intensity appeared to be relatively higher in the case of without crust than with crust. However, due to a greater variation in the case without crust (perhaps due to a larger spatial heterogeneity of water distribution), some data

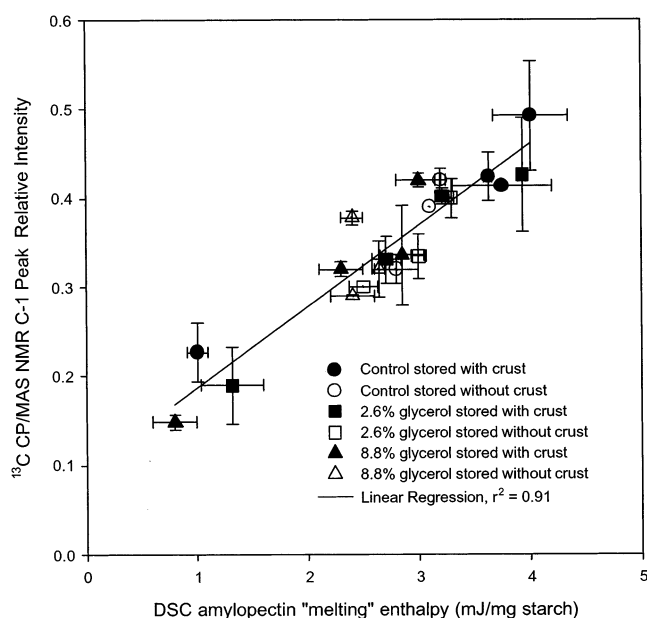


Figure 7. ^{13}C CP/MAS NMR C_1 intensity plotted against DSC amylopectin “melting” enthalpy for breadcrumbs containing various glycerol contents and stored with and without crust.

were not statistically significantly different. In addition, on the basis of the authors’ observation, the discrepancies seemed to be greater in the lower glycerol samples, but the data were not conclusive. The greater intensity might have been due to the higher level of rigid domains developed in starch (amylopectin), as observed previously from the DSC amylopectin “melting” endotherm around 40–80 °C, although this resulted in only very slight crystallization, as observed from peaks at 15 and 18° by X-ray diffraction (**Figure 3**), and a local moisture loss was observed in some domains by thermomechanical analysis (30).

Comparisons with DSC and Firmness Data. When stored with crust, breadcrumbs showed higher amylopectin melting enthalpy [as observed by DSC (20)] than breadcrumbs stored without crust, which could contribute to the higher ^{13}C CP/MAS NMR peak intensity. We plotted ^{13}C CP/MAS NMR peak intensities for all samples stored for different periods against the DSC endothermic enthalpy [data from (30)]. There was a fair correlation ($r^2 = 0.91$, **Figure 7**) but not a strong one, suggesting that the ^{13}C CP/MAS NMR resonance might also be contributed by other factors. The hydrogen-bond-breaking energy of the amylopectin helical structure contributes to the DSC endotherm. Hence, the increase in ^{13}C CP/MAS NMR spectral intensity was probably caused by some increase in molecular order and crystallization. But in some cases, when glycerol was present, it was found that the DSC endotherm was less than the control, but the relative ^{13}C CP/MAS NMR peak intensity was similar to the control (**Figure 6**). This suggests that other factors contribute to the backbone chain mobility.

The ^{13}C CP/MAS NMR intensity did not correlate as well with firmness data [data from (20)], with $r^2 = 0.68$ (data not shown). Because the bread staling process is a collection of events occurring simultaneously on multiple time scales and dimensions (i.e., from the molecular to the macroscopic level), it would not be appropriate to take an oversimplistic viewpoint by narrowing contributing factors to one or two components or events.

Glass Transition and Amorphous Rigid Components. The observed increase in ^{13}C CP/MAS NMR intensity could be partly related to a glassy transition (29) as chain mobility

decreased and more efficient cross-polarization occurred. The presence of glycerol leads to a major acceleration in the firming, as measured by an Intstron Universal Testing Machine (30), whereas ^{13}C CP/MAS NMR intensity and amylopectin enthalpy (20) decreased with addition of glycerol. This indicates that, while rigidity of starch decreased with added glycerol, firmness of bread increased at a more rapid rate. This suggested that firming of bread in this case was controlled by other events and not solely by starch retrogradation.

Although DSC and ^{13}C CP/MAS NMR showed similar patterns, data obtained from these two methods are not identical and thus not simply interchangeable. NMR results reflect combined retrogradation of starch, which includes helical structure formation, recrystallization, and loss of local water (rigid amorphous).

Previous reports (20, 30) and this work suggest that bread-crumbs firming in the presence of glycerol is mainly influenced by changes in amorphous polymer. Our data confirmed the suggestion by Hallberg and Chinachoti (9) that amylopectin recrystallization (DSC) and firmness of breadcrumbs might not increase in a parallel fashion (21).

In conclusion, ^{13}C CP/MAS NMR confirmed that there was a decrease in the rigidity of starch with glycerol addition and an increase in rigidity during storage, corresponding with reformation of a double-helical conformation. This was in agreement with DSC enthalpy results (20). However, addition of glycerol accelerated firming (based on our earlier report), suggesting that other mechanisms may be involved. We previously reported factors such as water distribution and migration as some of the important phenomena (8, 12, 20).

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