Biopolymer Interactions, Water Dynamics, and Bread Crumb Firming

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ABSTRACT: To establish the relationship between biopolymer interactions, water dynamics, and crumb texture evolution in time, proton mobilities in starch and gluten model systems and bread were investigated with NMR relaxometry. Amylopectin recrystallization was observed as an increased amount of fast-relaxing protons, while network strengthening and changes in water levels were noted as a reduced mobility and amount, respectively, of slowly relaxing protons. Amylopectin recrystallization strengthened the starch network with concomitant inclusion of water and increased crumb firmness, especially at the beginning of storage. The inclusion of water and the thermodynamic immiscibility of starch and gluten resulted in local gluten dehydration during bread storage. Moisture migration from crumb to crust further reduced the level of plasticizing water of the biopolymer networks and contributed to crumb firmness at longer storage times. Finally, we noted a negative relationship between the mobility of slowly relaxing protons of crumb polymers and crumb firmness.

KEYWORDS: *low-resolution proton nuclear magnetic resonance, proton mobility, amylopectin retrogradation, gluten hydration, water diffusion, bread firming*

■ INTRODUCTION

Wheat bread is an important staple food in the Western world. During storage, fresh bread loses a part of its desired texture and aroma associated with freshness. The crumb firms, the crust loses its crispiness, and the flavor of fresh bread disappears.[1] Although bread firming has been studied for a long time, its molecular basis is still not completely understood. Bread crumb is a sponge:[2,3] the solid part consists of a continuous phase composed of an elastic gluten network and leached amylose molecules and a discontinuous phase of (partially) gelatinized, swollen, and deformed starch granules.[4,5]

It is generally accepted that changes in the *starch fraction* during bread storage contribute to bread firming.[3,6−9] Amylose already crystallizes during cooling of the baked bread and contributes to initial crumb firmness.[8,10] Amylopectin recrystallization occurs over a longer time span.[4,10] However, there is no proof that the different starch properties alone can fully explain bread crumb firming.[10−14] Some authors suggest that *gluten*, the storage protein of wheat, which has unique dough-forming properties, also contributes to bread firming by cross-linking with starch granules through hydrogen bonding.[14] However, gluten may also have antifirming activity as it dilutes the starch.[1,15]

Not only the molecular order of starch but also changes in water content and distribution can increase bread crumb firmness.[1,16,17] Changes in *water content* during storage can be caused by two mechanisms. At a macroscopic level, water evaporates from bread. At the same time, on a molecular scale and over an extended time frame, the amorphous gelatinized amylopectin network of fresh bread crumb is transformed into a partially crystalline network. This network formation and crystallization of amylopectin impact the freezable water content in bread crumb[6,7,16] because water molecules become immobilized during storage by incorporation into B-type amylopectin crystals.[18−22] The crystalline hydrate water in the

B-type starch is structurally immobilized and unfreezable.[9,20,23,24]

Changes in *water distribution* during storage are caused by water migration from crumb to crust.[25−28] On a molecular scale, water is redistributed between gluten and starch. For one thing, water is incorporated into the formed B-type amylopectin crystals. However, whether or not this incorporated water fraction originates solely from the gluten network, as stated by some authors,[4,6,7,16,29] or also from the amorphous starch fraction itself has not yet been completely elucidated. Water migration from gluten to starch in bread can also be caused by diffusion.[7,30] When proteins and polysaccharides are mixed in water at high concentrations such as those prevailing in dough, they phase-separate in such a way that the polysaccharide phase is enriched in water. Thus, starch has a higher affinity for water than gluten. The thermodynamic immiscibility of gluten and starch can form the basis for diffusion of water from gluten to starch during storage of bread.[30] In contrast to amylopectin retrogradation, which has a maximal rate between 4 and 14 °C due to favored nucleation at temperatures far below the melting temperature of the crystals but above the glass transition temperature,[20,31] this diffusion process occurs faster at higher temperature.[7] Due to the high ratio of starch to gluten in bread crumb, moisture transfer from gluten to starch (due to retrogradation and/or diffusion) reduces the flexibility of the gluten network, which may contribute to crumb firmness.[6,7,16]

Molecular mobility of water and biopolymers in food products can be studied with low-resolution (LR) proton nuclear magnetic resonance (^{1}H NMR), which has been used for measuring transverse or spin−spin relaxation times (T_2) in

wheat biopolymer model systems[21,32−34] and bread.[17,21,33,35] In previous work, LR ^{1}H NMR was used to analyze proton mobilities in fresh bread crumb and crust based on the NMR profiles of starch, gluten, and flour model systems analyzed before and after heat treatment.[33] Proton relaxation curves were transformed into a continuous distribution of T_2 relaxation times. By comparing the bread crumb proton distributions with those of the biopolymer model systems, protons in fresh crumb were divided into five populations of different mobility depending on the local environment. The lowest T_2 and thus most rigid fraction contains rigid CH protons of amylose crystals formed during cooling and of amorphous starch and gluten not in contact with water. With increasing proton mobility, the populations consist of CH protons of amorphous starch and gluten in little contact with water and CH protons of gluten and exchanging protons of confined water, starch, and gluten. The largest population contains mobile exchanging protons of water, starch, gluten, and minor components in the formed gel network. Finally, a small lipid proton population has the highest mobility (highest T_2). With the distinction between the different molecular environments, changes in proton distributions can be linked to molecular and physical changes during storage. With magnetic resonance imaging, water migration between gluten and arabinoxylans in bread dough was observed.[36]

The objective of this study is to establish the relation between biopolymer interactions, water dynamics, and changes in bread texture during storage. Focus will be on the molecular changes in starch and gluten and the status of water in crumb and crust.

■ MATERIALS AND METHODS

Materials and Compositional Analysis. Wheat flour (Surbi) [71.5% starch, 11.1% protein, 13.9% moisture content (MC)] was provided by Dossche Mills (Deinze, Belgium). Wheat starch (25.8% amylose, 11.2% MC) and wheat gluten (10.6% starch, 78.0% protein, 8.0% MC) were from Tereos Syral (Aalst, Belgium). Starch content was determined by gas−liquid chromatography as in Courtin and Delcour.[37] Protein content was determined by an adaptation of the AOAC official method[38] to an automated Dumas protein analysis system (EAS vario Max C/N, Elt, Gouda, The Netherlands), with 5.7 as the nitrogen to protein conversion factor. MC of flour, starch, gluten, model systems, and bread crumb and crust was determined according to AACC method 44-15.02.[39] Amylose content was determined according to the Megazyme (Bray, Ireland) procedure, which itself is based on Yun and Matheson.[40] All reagents, solvents, and chemicals were of analytical grade and obtained from Sigma−Aldrich (Bornem, Belgium) unless indicated otherwise.

Preparation of Model Systems. Wheat starch and gluten model systems, both with water content of 47% and thus representing a typical bread dough MC, were analyzed with LR ^{1}H NMR and differential scanning calorimetry (DSC; see below) after heat treatment in an oil bath (10 min at 110 °C) with subsequent cooling to room temperature for 2 h and during storage at 25 °C for 48, 120, and 168 h. The NMR tubes were filled to a height of 8 mm (approximately 0.3 g of sample). The starch model system was also analyzed with ^{1}H NMR and DSC after reheating in the oil bath (same conditions) and subsequent cooling to room temperature for 3 h. The heated gluten model system was also analyzed with ^{1}H NMR after drying to MC of 36% with forced air (temperature 24 °C, hair dryer). This MC corresponded to that of crumb of bread stored for 168 h (see below). The samples were (re)heated (oil bath) and dried (forced air) in NMR tubes. The sample tubes were analyzed in triplicate (see below).

Bread Making. Bread was made according to a straight-dough method[41] for 100 g of flour. Dough ingredients [100.0 g of wheat flour

(14.0% moisture), 5.3 g of compressed yeast (AB Mauri, Merelbeke, Belgium), 6.0 g of sucrose, 1.5 g of NaCl, 57.0 mL of water, and 0.25 g of calcium propionate] were mixed for 240 s in a 100 g pin mixer (National Manufacturing, Lincoln, NE) at 25 °C and fermented in a fermentation cabinet (National Manufacturing) for 90 min (30 °C, 90% relative humidity) with intermediate punching at 52 and 77 min and final punching at 90 min using a dough sheeter (National Manufacturing). Following molding and proofing (36 min at 30 °C, 90% relative humidity), dough was baked at 215 °C for 24 min in a rotary oven (Model Hearth, National Manufacturing). Prior to further analyses, breads were cooled for 1−2 h.

Storage of Bread. After the freshly baked breads were cooled for 1−2 h, three samples from the center crumb of different breads and three samples from the top center crust of different breads were placed in separate sealed NMR tubes. These tubes were stored at 25 °C and analyzed by ^{1}H NMR during storage after 2, 48, 120, and 168 h. In this way, the effect of amylopectin retrogradation on the proton distributions in bread crumb during storage could be studied without interference of water migration from crumb to crust. At the same time, fresh whole breads were wrapped in plastic foil and stored at 25 °C for 168 h in sealed plastic bags to prevent moisture loss. The additional effect of water migration from crumb to crust on the proton distributions during storage could now be studied.

Drying of Fresh Bread Crumb. Cylindrical samples (such as made for texture analysis; see below) were cut from the center of bread, immediately after it was cooled for 1−2 h, and air-dried at 25 °C. After 0, 1, 2, and 3 h of drying, compression and DSC analyses were performed. For the ^{1}H NMR measurements, forced air (temperature 24 °C, hair dryer) was used to dry samples in NMR tubes to approximately the same MC as bread crumb stored with crust for 168 h (MC 36%) and as bread crumb air-dried for 3 h (MC 24.4%). Samples were then prepared with moisture loss but without significant amylopectin retrogradation.

Differential Scanning Calorimetry. DSC was performed with a Q1000 DSC (TA Instruments, New Castle, DE). The starch−water model system was analyzed as such, while bread crumb was freeze-dried after different storage days for analyzing retrograded amylopectin. The (freeze-dried) samples were accurately weighed (2.5−4.0 mg) in triplicate in aluminum pans (Perkin-Elmer, Waltham, MA). Deionized water was added in a ratio of 1:3 [w/w, sample dry matter (DM):water]. The pans were hermetically sealed and equilibrated at 0 °C before being heated to 120 °C at 4 °C/min (together with an empty reference pan). Before analysis, the system was calibrated with indium. The temperatures and enthalpies corresponding to the melting of amylopectin crystals (retrogradation) were evaluated from the thermograms by use of TA Instruments Universal Analysis software. Enthalpies were expressed in joules per gram of sample (on DM basis).

Besides the extent of amylopectin retrogradation, the amount of freezable water (FW, the water that transforms into ice during cooling to −40 °C; see below) in bread crumb was determined. Bread crumb (10−15 mg) was accurately weighed in aluminum pans. The samples were equilibrated at 15 °C and cooled to −40 °C at a rate of 4 °C/min, held for 5 min at −40 °C, and reheated to 30 °C at 4 °C/min. From the melting enthalpy, measured between −6 and 0 °C, and the MC of the sample, the amount of FW was calculated as

$$\%\text{FW} = \frac{\Delta H_{\text{melting}}}{\Delta H_{\text{ice}} \times \text{MC}} \times 100$$

where $\Delta H_{\text{melting}}$ is the melting enthalpy of ice in the sample (joules per gram of sample, on as-is basis); ΔH_{ice} is the melting enthalpy of ice (334 J/g of ice), and MC is expressed in grams of water per gram of sample.[42]

To determine the FW content of fresh bread crumb, three pans were analyzed between 2 and 12 h after baking. The other pans were stored at 25 °C and analyzed after 48, 120, and 168 h.

Wide-Angle X-ray Diffraction. A Rigaku (Tokyo, Japan) high-temperature X-ray diffractometer was used to perform wide-angle X-ray diffraction (WAXD) measurements with Ni-filtered Cu $\kappa\alpha$
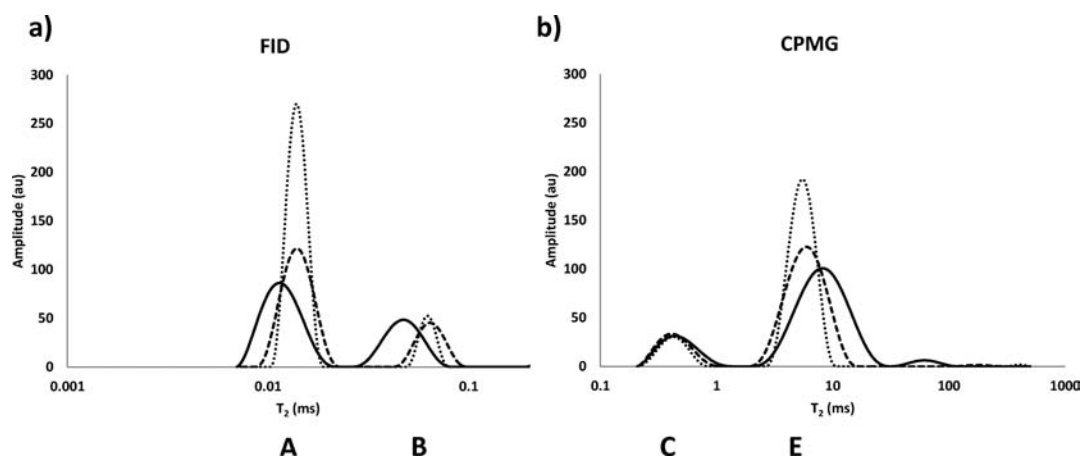
Figure 1. (a) FID and (b) CPMG proton distributions of a heated (——), stored (···), and reheated (---) wheat starch−water model system, obtained by inverse Laplace transformation of the FID and CPMG pulse sequence. Amplitudes are given in arbitrary units (au). The different proton populations are indicated with capital letters in order of their increasing mobility as described in Bosmans et al.[33].

radiation on a rotating anode device operating at 40 kV and 100 mA. Detection was with a scintillation counter with a powder diffractometer operating in transmission mode. Fresh and stored bread crumb were placed as such in a sample holder and covered on both sides with aluminum foil. Scattering patterns were measured in 3−40° 2θ range with a fixed time of 10 s/step of 0.05° 2θ.

Crumb Texture Analysis. Firmness of bread crumb was measured during storage and drying by compression with an Instron (Norwood, MA) 3342. From the center of fresh and stored bread, cylindrical samples (height 25 mm, diameter 30 mm) were cut with a sharp borer. A cylindrical probe with a diameter of 75 mm compressed the samples at a constant test speed of 100 mm/min. The force required to compress samples by 30% was determined from the force−time curve and is further referred to as firmness.

^{1}H NMR. Measurements of proton distributions in the model systems and bread were performed with a Minispec mq 20 low-field pulsed NMR spectrometer (Bruker, Rheinstetten, Germany), with an operating resonance frequency of 20 MHz for [1]H (magnetic field strength of 0.47 T). The probe head was kept at 25 ± 1 °C. An external water bath maintained the desired temperature. T_2 values were determined. The relaxation curves were acquired by use of subsequently a single 90° pulse (free induction decay, FID)[43] and a Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence.[44] The pulse lengths of the 90° pulse and 180° pulse were 2.86 and 5.42 μs, respectively. For the FID signal, an acquisition period of 0.5 ms was used and 500 data points were acquired. For the CPMG sequence, the pulse separation between the 90° and 180° pulse was 0.1 ms and 2500 data points were collected. For both measurements, a recycle delay of 3.0 s was used and 32 scans were accumulated to increase the signal-to-noise ratio.

Samples of the unheated model systems, crumb, and crust (approximately 0.3 g, accurately weighed) were placed in three Bruker NMR tubes (10 mm external diameter) and tightly compressed to a height of 8 mm to avoid large air holes. The tubes were sealed to prevent moisture loss. Each tube was analyzed in triplicate, and the exact sample weight in each tube was taken into account.

The CONTIN algorithm of Provencher[45] (software provided by Bruker) was used to transform the transverse relaxation curves with an inverse Laplace transformation to continuous distributions of T_2 relaxation times. In the FID experiments, less mobile protons are analyzed, while the more mobile protons are detected with the CPMG pulse sequence. The areas under the curves of the populations with certain T_2 relaxation times are proportional to the number of protons in the population. For both the model systems and bread, the inhomogeneity of the static magnetic field affected the output for the most mobile FID population (around 0.5 ms). Therefore, this population was not taken into account in the following analyses.

RESULTS AND DISCUSSION

Biopolymer Model Systems. The changes in proton distributions during heating and subsequent cooling of a
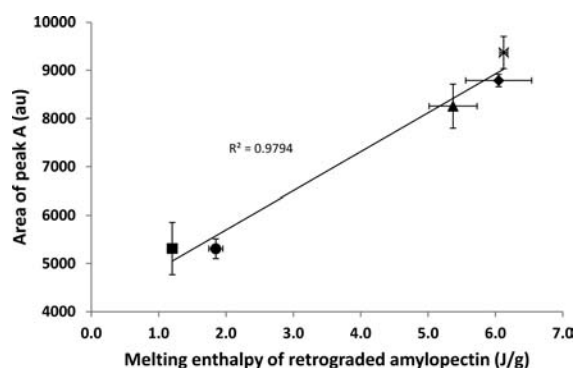

Figure 2. Area of the first FID population (population A), representing solid CH protons of starch crystals and densely packed amorphous starch not in contact with water (measured by NMR), versus melting enthalpy of retrograded amylopectin (measured by DSC) of a heated (10 min at 110 °C) starch−water model system measured after storage for 2 (■), 48 (▲), 120 (◆), and 168 (✕) h and after reheating with subsequent cooling of 3 h (●). It can be seen that area and melting enthalpy are linearly related ($R^2 = 0.9794$).

starch−water model system (MC of 47%) have been described by Bosmans et al.[33] Figure 1 shows the proton distributions of the heated, stored, and reheated model system. Immediately after cooling, population A contained rigid nonexchanging CH protons of amylose crystals formed during cooling and/or amorphous starch that remained immobile after heating, while both populations B and C were assumed to contain the same amorphous CH protons.[33] During storage of the starch gel, the area of population A, representing inter alia starch crystal protons, increased, while the areas of populations B and C, representing amorphous starch protons, decreased (Figure 1). When the area of population A was plotted against the DSC melting enthalpy of retrograded amylopectin, a clear relationship could be observed (Figure 2). Hence, it seems plausible that amorphous amylopectin CH protons from populations B and C, which became incorporated into crystalline structures during storage, lost mobility and became part of population A. This polymer reorganization on a molecular scale was indeed

Table 1. Moisture Content, Crumb Firmness, Freezable Water, and Melting Enthalpy of Retrograded Amylopectin of Bread Crumb during 168 h of Storage at 25 °C[a]

storage time (h)	MC (%)		crumb firmness (N)	FW (%)	ΔH_{AP} [J/g crumb (DM)]
	crumb	crust			
2	43.4 (0.1)	16.3 (0.0)	1.1 (0.2)	47.0 (4.1)	0.25 (0.02)
48	41.5 (0.3)	18.5 (0.2)	2.4 (0.1)	48.0 (1.3)	2.03 (0.06)
120	37.7 (0.6)	23.6 (0.6)	4.8 (0.2)	39.9 (4.5)	3.26 (0.18)
168	36.4 (0.7)	23.0 (0.0)	7.0 (0.5)	38.5 (1.7)	3.34 (0.22)

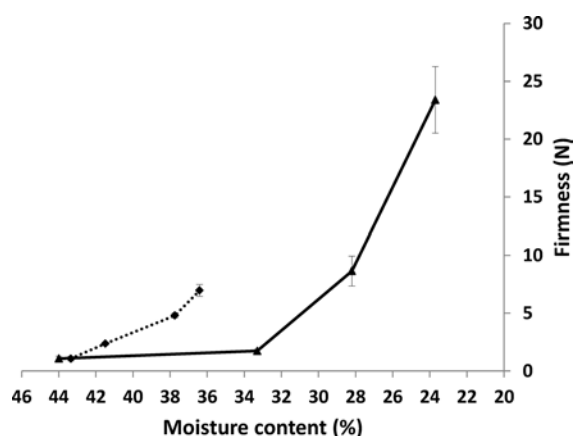[a]Standard deviations are given in parentheses.

Figure 3. Firmness as a function of moisture content, obtained by drying (——) and storage (···) of bread for 3 and 168 h at 25 °C, respectively.
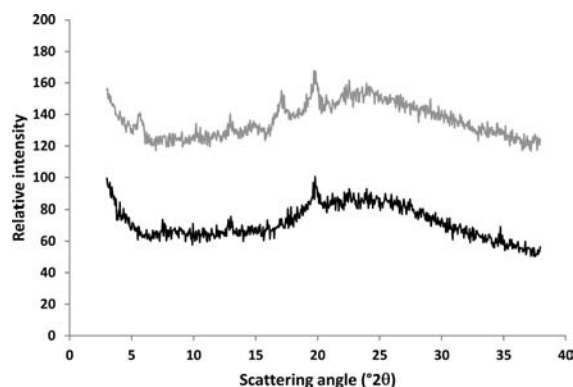

Figure 4. X-ray diffractograms of fresh (black trace) and stored (168 h, gray trace) bread crumb.

observed as a concomitant reduction in areas of populations B and C (Figure 1). The areas decreased from 3169 to 1397 au and from 1894 to 1197 au, respectively. The large and broad CPMG population E, which contains exchanging protons of starch and water in the formed gel network during cooling,[33] became more homogeneous (reduced peak width) and less mobile (reduced T_2) upon storage of the starch gel (Figure 1b). These observations can be explained by a strengthening of the gel network due to amylopectin retrogradation. The starch crystallites formed during cooling (amylose) and storage (amylopectin) create network junction zones, which serve as nucleation sites for developing an intermolecular and maybe even intergranular network.[46] Such network can be presented as a fringed micelle network.[20,47,48] In this network, amylose and amylopectin molecules pass through more than one ordered, crystalline region, resulting in micelles formed by

aggregation of a number of different molecules over a particular region of the chain that are linked to each other by amorphous regions.[48] Figure 1 allows us to conclude that the molecular and mesoscale events related to amylopectin retrogradation can clearly be detected in a nondestructive way with ^{1}H NMR as a shift in proton populations toward lower mobility, whereby an increase in area of the rigid population A points to formation of amylopectin crystals, while the reduced mobility of the mobile population E is probably the result of starch network strengthening due to an increased amount of network junction zones during storage.

When the starch gel was reheated after storage and then allowed to cool to room temperature, the proton distributions changed. The area of population A decreased, while the areas of populations B and C increased simultaneously (Figure 1). DSC showed a decrease in melting enthalpy of the amylopectin crystals to a value comparable to that of a fresh starch gel, showing that the crystals formed during storage were melted during reheating (Figure 2). The melting of amylopectin crystals was also observed as a decrease in the area of population A, which mostly contained the solid protons present in a crystalline environment, and the associated increase in areas of populations B and C (Figure 1), which contained the more mobile, amorphous CH protons. Reheating increased the peak width and mobility (T_2) of population E of the stored starch gel, but not to the values for peak width and T_2 of population E in the fresh starch gel (Figure 1b). This population with exchanging protons of starch and water in the gel network formed lost mobility due to formation of a continuous, intermolecular, partially crystalline, permanent network. The increase in its heterogeneity after reheating demonstrated that this network had become more mobile due to loss of crystallinity but also that the protons of the network are still in a less mobile environment than in the case of a fresh starch gel. The latter indicates that reheating the stored gel does not fully reverse the network organization of starch to that of a fresh gel.

When the heated gluten–water model system was stored, no large changes in proton distributions were observed (results not shown). Thus, in the absence of other biopolymers, neither changes in the gluten gel nor changes in its internal water distribution occurred during storage.

Bread. *Physical Changes during Storage.* During 168 h of bread storage at 25 °C, the crumb MC decreased from 43.4% to 36.4%, while that of the crust increased from 16.3% to 23.0% (Table 1). At the end of storage, the moisture migration between crumb and crust leveled off. Crumb firmness increased almost linearly during storage (Table 1).

To verify the impact of crumb water loss on its mechanical properties, crumb was dried at ambient conditions in a short time frame. During drying, no change in crumb firmness was detected above a crumb MC of 33% (Figure 3). Further drying
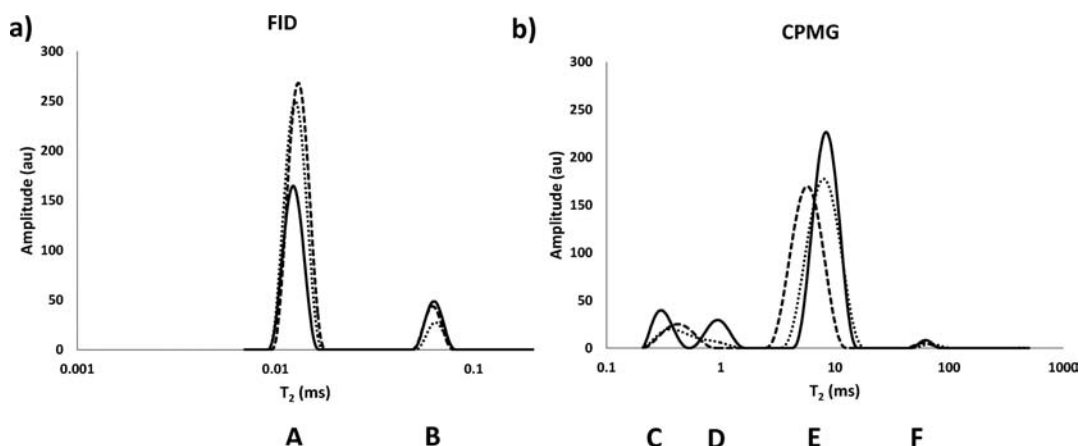
Figure 5. (a) FID and (b) CPMG proton distributions of fresh bread crumb (—), bread crumb removed after 1−2 h of cooling and stored for 168 h in NMR tubes (···), and bread crumb removed after storage of whole breads (with crust) for 168 h and placed in NMR tubes prior to analysis (---), obtained by inverse Laplace transformation of the FID and CPMG pulse sequence. Amplitudes are given in arbitrary units (au). The different proton populations are indicated with capital letters in order of their increasing mobility.

Table 2. Relaxation Times and Areas of Populations A, D, and E of Bread Crumb during 168 h of Storage in NMR Tubes[a] at 25 °C[b]

storage time (h)	population A (FID)		population D (CPMG)		population E (CPMG)	
	T_2 (ms)	area (au)	T_2 (ms)	area (au)	T_2 (ms)	area (au)
2	0.014 (0.002)	4885 (462)	1.07 (0.15)	986 (61)	8.30 (0.18)	8961 (34)
48	0.014 (0.000)	6885 (378)	0.97 (0.15)	760 (129)	7.92 (0.07)	8960 (31)
120	0.014 (0.000)	7611 (28)	0.95 (0.07)	403 (110)	7.60 (0.03)	9044 (25)
168	0.013 (0.001)	8556 (461)	1.05 (0.07)	413 (59)	7.56 (0.16)	8978 (77)

[a]That is, no crumb to crust moisture migration. [b]Population A, rigid CH protons of starch crystals and amorphous starch and gluten not in contact with water; population D, CH protons of gluten and exchanging protons of confined water, starch, and gluten; population E, mobile exchanging protons of water, starch and gluten in the formed gel network. Standard deviations are given in parentheses.
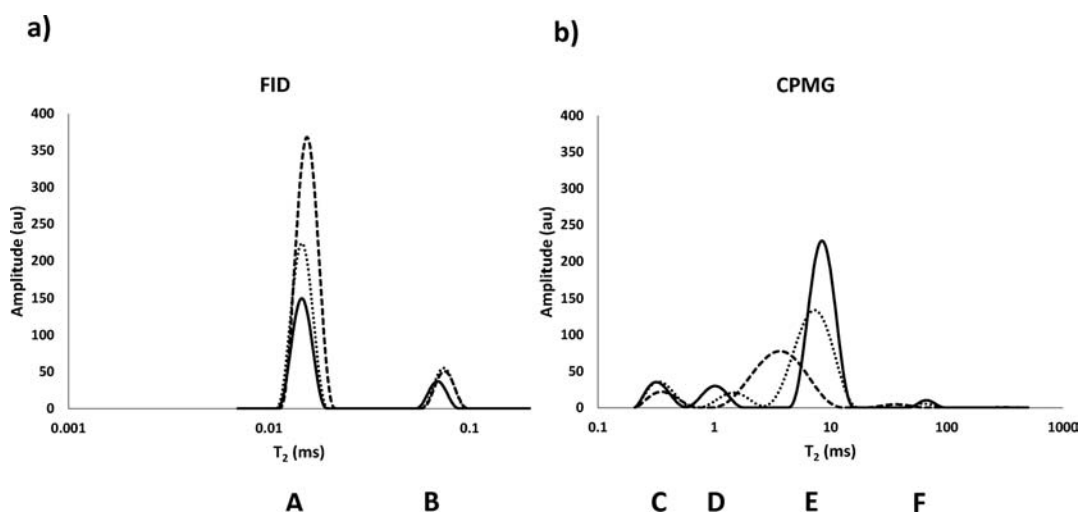

Figure 6. (a) FID and (b) CPMG proton distributions of fresh bread crumb with 44.3% moisture content (MC, ——), fresh bread crumb with 36.8% MC (···), and fresh bread crumb with 24.4% MC (---) obtained by inverse Laplace transformation of the FID and CPMG pulse sequence. Amplitudes are given in arbitrary units (au). The different proton populations are indicated with capital letters in order of their increasing mobility.

of fresh bread crumb below that MC resulted in a strong increase in firmness (Figure 3). The melting enthalpy of amylopectin crystals did not exceed a value of 0.35 J/g, showing that no significant amylopectin retrogradation had occurred upon drying of the bread crumb to a MC of 24% over a short time period. Apparently, the biopolymer networks in bread crumb were fully plasticized above MC of about 33%. Additional water did not contribute to the mechanical

properties of crumb. Gluten amide groups are fully hydrated at a water content of about 35%. Addition of water above this MC results in dilution instead of further hydration of the proteins.[49] When these literature findings are confronted with the present results, it seems reasonable that below a crumb MC of 33%, further withdrawal of water stiffened the gluten strands due to loss of plasticizer and caused an increase in firmness. A similar reasoning may also be valid for the starch network, since
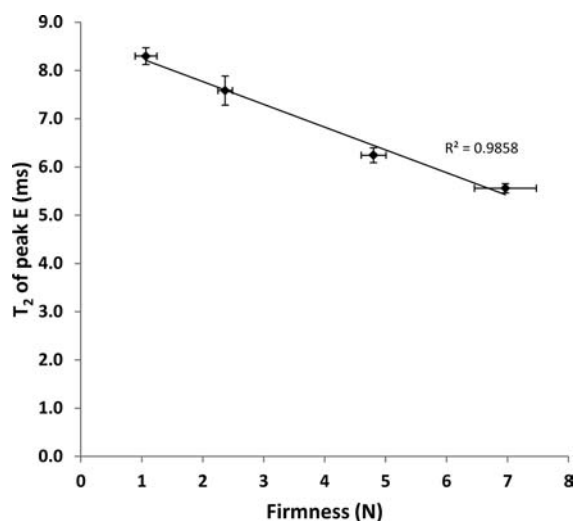
Figure 7. Relaxation time (T_2) of the most mobile CPMG population E, representing mobile exchanging protons of water, starch, and gluten present in the formed gel network (measured by NMR), versus firmness (measured by texture analysis) of bread crumb stored with crust at 25 °C. It can be seen that T_2 and firmness are linearly related ($R^2 = 0.9858$).

the amorphous matrix in a retrograded wheat starch gel appears to be fully plasticized at MC of about 27%.[47] In this study, the MC of crumb was well above the level for fully plasticizing gluten strands and the starch matrix during storage of bread. Hence, it would be expected that crumb to crust moisture migration did not contribute greatly to the observed increase in crumb firmness. The amount of FW decreased during storage (Table 1), even when no loss in crumb MC had occurred.

To further elucidate the molecular dynamics behind the increased firmness and the decrease in FW during storage, amylopectin crystal formation (DSC and WAXD measurements) and changes in proton distributions (^{1}H NMR measurements) in bread crumb were studied.

Molecular Changes during Storage. An increase in melting enthalpy of retrograded amylopectin in crumb with time was observed with DSC (Table 1), showing the formation of amylopectin crystals during storage. Most of the crystals were formed during the first days, and only a small increase in enthalpy was detected after a storage period of 120 h (Table 1). The formed starch crystals had a B-type X-ray diffraction pattern, which is characterized by diffraction angles at 5.6, 15.0, 17.2, 22.4, and 24.1° 2θ (Figure 4).[50] Compared to A-type crystals of native wheat starch, B-type crystals have more water molecules in their structure.[22] When water is incorporated into the B-type amylopectin crystals, it becomes structurally immobilized and is thus rendered unfreezable.[20] This was observed with DSC as a decrease in FW during storage (Table 1). The maximum water content of B-type crystals formed during storage is 27% (w/w).[51] When B-type starch crystallites, prepared by acid hydrolysis of potato starch for 20 days at 35 °C,[52] were measured with DSC, the melting enthalpy was 70 J/g DM sample (results not shown). On the basis of these assumptions and the actual amount of crystals formed (observed with DSC), it was calculated that about 2−3% of all water present in crumb is immobilized into the amylopectin crystals. The calculated values also correspond to values found in literature.[21] However, the amount of water that became unfreezable during storage was about 8% (Table 1). This indicates that, besides incorporation of water into the formed B-type crystals, other phenomenona also decreased the FW content. During bread storage, amylopectin side chains reorganized, presumably into both intra- and intermolecular crystallites. These crystals served as junction zones between amorphous regions, resulting in a continuous intergranular fringed micelle starch network[20,47,48] that extended throughout the bread crumb.[10,16] It is hypothesized that this intergranular fringed micelle network included an additional water fraction during storage and rendered it unfreezable.

Crumb firmness increased linearly (Table 1), while amylopectin retrogradation and the decrease in FW leveled off at the end of storage (Table 1). Another factor, besides reinforcement of the starch network and the concomitant inclusion of water, must have contributed to the increase in crumb firmness at a later stage. At the end of storage, crumb MC decreased to a value of 36.4% (Table 1). This water content was still high enough to keep the amorphous networks plasticized in fresh bread crumb (Figure 3). However, during storage, amylopectin retrogradation also occurred with
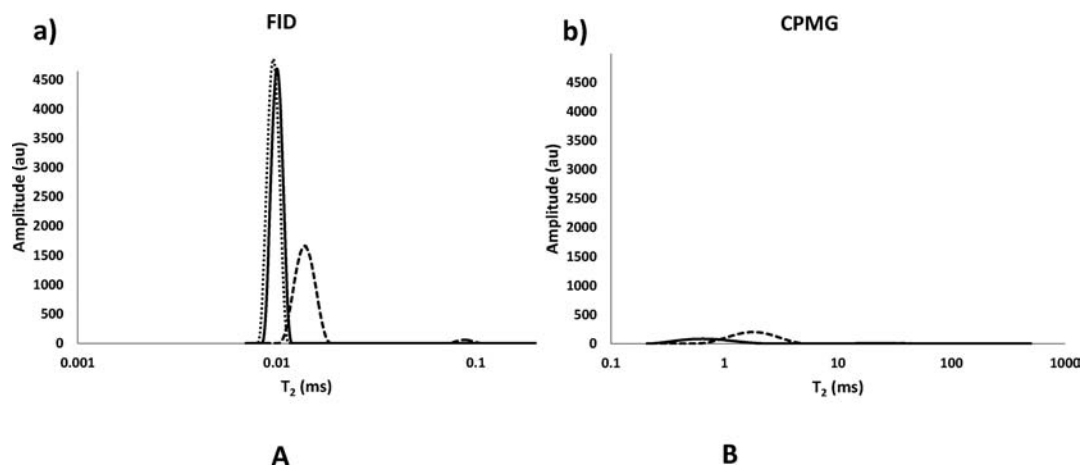

Figure 8. (a) FID and (b) CPMG proton distributions of fresh bread crust (-), bread crust removed after 1−2 h of cooling and stored for 168 h in NMR tubes (...), and bread crust removed after storage of whole breads (with crust) for 168 h and placed in NMR tubes prior to analysis (---), obtained by inverse Laplace transformation of the FID and CPMG pulse sequence. Amplitudes are given in arbitrary units (au). The different proton populations are indicated with capital letters in order of their increasing mobility.

formation of an intermolecular and maybe even intergranular starch network (see above). Provided that inclusion of water due to retrogradation together with crumb to crust moisture migration reduced the MC of the biopolymer networks in crumb below the critical value necessary for full plasticization, the resultant increase in stiffness of these networks would have contributed to crumb firmness, especially at the end of the storage period. This hypothesis was further investigated by using ^{1}H NMR.

With ^{1}H NMR, six different proton populations could be distinguished in fresh bread crumb (Figure 5). Population A consists of rigid CH protons of amylose crystals and amorphous starch and gluten not in contact with water. Populations B and C represent the same protons and contain more mobile CH protons from amorphous starch and gluten in little contact with water. Population D contains CH protons of gluten and exchanging protons of confined water, starch, and gluten. Population E consists of mobile exchanging protons of water, starch, and gluten in the formed gel network. The most mobile population, F, contains lipid protons.[33] When fresh bread crumb was stored without crust in sealed NMR tubes for up to 168 h, evidently no crumb to crust moisture migration occurred. Changes in proton distributions could therefore be attributed to changes in the starch network, such as those resulting from amylopectin retrogradation. The latter was measured with DSC (Table 1). The area of population A increased (Figure 5a and Table 2), while the areas of populations B and C decreased (Figure 5). Furthermore, the area of population D decreased and the mobility (T_2) of population E decreased (Figure 5b and Table 2). The increase in area of population A and the decrease in areas of populations B and C were also observed in the starch–water model system and were attributed to formation of amylopectin crystals, which resulted in a shift of protons to population A with lower mobility (see above, Figures 1 and 2 and Table 2). In line with the results for the starch model system, the reduced mobility of the protons in population E (reduced T_2) was attributed to strengthening of the starch network due to amylopectin retrogradation (Figures 1b and 5b). The reduction in area of population D showed that some CH protons of gluten and exchanging protons of confined water, starch, and gluten ended up in a less mobile environment and therefore shifted to population C. Due to this shift, populations C and D eventually merged (Figure 5b and Table 2). The merging of populations C and D and the reduction in T_2 of population E were also observed in a gluten–water model system that was gradually dried (results not shown). Hence, the change in environment (reduction in area of population D) and reduced mobility (reduced T_2 of population E) can be related to loss of water from the gluten network in bread. As there was neither crumb to crust migration nor water evaporation in these samples, this supports the hypothesis of water diffusion from gluten to starch due to the immiscibility of these polymers.[7,30] Because population D contained CH protons of gluten and exchanging protons of confined water, starch, and gluten, it is conceivable that reduction of the amount of water associated with the gluten strands contributed to a shift of the gluten protons to population C. Possibly the decreased mobility of the water fraction in population D, reflected in a reduction of the level of FW, contributed to the merging of populations C and D. Exchangeable protons of gluten were also present in population E, so a loss of water from the gluten network possibly contributed to the reduction in T_2 of this population. When

fresh bread crumb was dried to approximately the same MC as bread crumb stored for 168 h with crust (36.8%), a reduction in proton mobility could also be observed (Figure 6). Since little if any amylopectin retrogradation took place in these samples as observed with DSC (see above), the changed proton mobility was related only to the reduction in MC. The largest changes were an increase in area of population A and a decrease in area and T_2 of population E. Although this decrease in MC did not contribute to crumb firmness (see above, Figure 3), it led to a change in proton distributions. Remarkably, in contrast to what was observed for crumb samples stored for 168 h in NMR tubes (Figure 5b), no merging of populations C and D occurred in the dried bread crumb (Figure 6b). Apparently, the MC in the dried sample (36.8%) was still high enough for the gluten network to be fully plasticized. This is in line with the literature about gluten hydration[49] and also suggests strong local gluten dehydration during bread storage, probably as a result of starch retrogradation and/or water diffusion to starch. When bread crumb was dried further to a MC of approximately 24%, without significant amylopectin retrogradation, the proton mobility was largely reduced (Figure 6). Due to the low MC, the area of population E decreased to a large extent and its mobility (T_2) was reduced further (Figure 6b). The area of population A greatly increased, pointing to more protons being in a very rigid environment. A distinction between populations C and D could no longer be made, which indicates gluten dehydration as a result of the water level of the crumb dropping below 35% (Figure 6b).

When bread was stored with crust, both amylopectin retrogradation and crumb to crust moisture migration occurred. This resulted in an increase in area of population A after 168 h of storage, while the areas and T_2 relaxation times of populations D and E now decreased to a larger extent than those of the crumb samples stored in NMR tubes (Figure 5). A strong linear relationship was found between the increase in crumb firmness and the decrease in T_2 of population E during storage of bread as a whole (Figure 7). Hence, from the NMR profiles of bread crumb, not only the extent of formation of amylopectin crystals (area of population A, Figure 2) but also the extent of crumb firmness (T_2 of population E, Figure 7) can be deduced. The reduced area of population E in crumb stored with crust resulted from the reduction in crumb MC.

The NMR profiles of bread crust removed directly after cooling and stored in sealed tubes for 168 h did not change over time (Figure 8). Starch in the crust is not gelatinized and therefore evidently cannot retrograde. Due to the low MC of crust (16.3%, Table 1), most of its protons were present in the solid FID population A (Figure 8a). When crust samples were removed after storage of whole bread and then analyzed, the area of population A was lower and its T_2 was higher than that of fresh crust (Figure 8a). At the same time, the area and T_2 of population B were higher (Figure 8b), showing that the mobility of different crust proton populations increased during storage. This can be explained by the higher MC of the stored crust than that of fresh crust (23.0% and 16.3%, respectively; Table 1) due to moisture migration.

It can be concluded that the increase in bread crumb firmness is caused by a combination of different events. Amylopectin retrogradation and the formation of a continuous, rigid, crystalline starch network that includes water in its structure were strongly related to the increase in crumb firmness of stored bread, especially during the first days of storage. The inclusion of water in the starch network withdrew

water from the amorphous networks in bread crumb and reduced the amount of FW. The thermodynamic immiscibility of starch and gluten resulted in moisture migration from gluten to starch. During bread storage, water also migrated from crumb to crust, leading to an additional reduction in MC of the gluten network. After a couple of days of storage, when this MC had dropped below the critical point for gluten to be fully plasticized, the resulting increase in stiffness contributed to the increase in crumb firmness. On the basis of changes in NMR profiles of bread crumb, an estimation of the extent of amylopectin crystal formation and of firmness could be made.

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Notes

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■ ABBREVIATIONS

LR, low-resolution
^{1}H NMR, proton nuclear magnetic resonance
T_2, spin–spin relaxation time
MC, moisture content
DSC, differential scanning calorimetry
WAXD, wide-angle X-ray diffraction
DM, dry matter
FW, freezable water
$\Delta H_{melting}$, melting enthalpy of ice in the sample
ΔH_{ice}, melting enthalpy of ice
° 2θ, scattering angle
FID, free induction decay
CPMG, Carr–Purcell–Meiboom–Gill
au, arbitrary units
ΔH_{AP}, melting enthalpy of retrograded amylopectin

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