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Impact of Amylases on Biopolymer Dynamics during Storage of Straight-Dough Wheat Bread

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ABSTRACT: When *Bacillus stearothermophilus* α-amylase (BStA), *Pseudomonas saccharophila* α-amylase (PSA), or *Bacillus subtilis* α-amylase (BSuA) was added to a bread recipe to impact bread firming, amylose crystal formation was facilitated, leading to lower initial crumb resilience. Bread loaves that best retained their quality were those obtained when BStA was used. The enzyme hindered formation of an extended starch network, resulting in less water immobilization and smaller changes in crumb firmness and resilience. BSuA led to extensive degradation of the starch network during bread storage with release of immobilized water, eventually resulting in partial structure collapse and poor crumb resilience. The most important effect of PSA was an increased bread volume, resulting in smaller changes in crumb firmness and resilience. A negative linear relation was found between NMR proton mobilities of water and biopolymers in the crumb and crumb firmness. The slope of that relation gave an indication of the strength of the starch network.

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

INTRODUCTION

Firming of bread is mainly related to changes in the starch fraction and formation of a continuous, rigid, crystalline starch network.[1−4] On a molecular scale, amylose crystallizes during cooling immediately after baking, while amylopectin over a longer time span recrystallizes in a process referred to as retrogradation.[2,5−7] The amylose and amylopectin crystallites create network junction zones. These zones serve as nucleation sites for an intermolecular and maybe even intergranular mesoscale network[7] that includes unfreezable water.[8] Such network can be presented as a fringed micelle starch network[9−11] in which amylose and amylopectin molecules pass through more than one ordered, crystalline region, resulting in micelles formed by aggregation of different molecules over a particular region of the chain, which are linked to each other by amorphous regions.[11]

The fast-forming amylose network and the thermoset gluten network are largely responsible for initial crumb firmness and resilience, while amylopectin retrogradation strengthens the network during bread storage.[7] Part of the water included in the continuous starch network is withdrawn from the amorphous networks in bread crumb, which, therefore, lose plasticizing water. Furthermore, water diffuses from gluten to starch due to their thermodynamic immiscibility.[8,12] Besides water redistribution within bread crumb during storage, water also migrates from crumb to crust, leading to further local reduction in moisture content of the biopolymer networks. As a result, the moisture content of the gluten network can drop below that needed for full plasticization during storage. The resulting increase in stiffness of the gluten network then also contributes to crumb firming, mostly after a couple of days of storage.[8] Taken together, these changes are responsible for the rather short shelf life of bread.

Several amylases and, more in particular, *Bacillus stearothermophilus* maltogenic α-amylase (BStA), decrease the rate of bread firming.[6,7,13] Their impact depends inter alia on the temperature window of their activity during baking, the used dosage, and their mode of action.[13] However, the exact mechanism by which amylases decrease crumb firmness is still under debate. It has been suggested that their antifirming properties are related to their ability to partially degrade amylose and amylopectin, resulting in changes in crystallization (in the case of amylose) and recrystallization (in the case of amylopectin) behavior of these polymers during cooling and storage, respectively.[6,13,14]

Amylases display either an endo- or an exomode of action. Endo-α-amylases such as *Bacillus subtilis* α-amylase (BSuA) mainly hydrolyze starch polymers internally. BSuA displays a low degree of multiple attack (DMA), that is, the number of bonds cut, following the first one, without dissociation of the enzyme−substrate complex.[15−17] Its action results in a fast reduction of the starch molecular weight[13,17] and a softer bread crumb.[13] However, because this enzyme can still be active after baking, it can cause extensive degradation and (partial) structure collapse.[13] In contrast, BStA is best described as an exoamylase, which remains attached rather long to the same starch chain (high DMA), resulting in a slow reduction of the overall starch molecular weight.[13,17,18] The current hypothesis is that its action produces amylopectin side chains that are too short to recrystallize, which therefore results in decreased bread crumb firmness.[13] At higher temperatures, BStA also displays some endoaction,[15,17] which may result in an additional weakening of

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the starch network. It reduces the molecular weight of amylose such that the polymer more effectively forms an amylose network during cooling.[6,13,19] A third interesting enzyme in the context of breadmaking is a maltotetraogenic, that is, a maltotetraose forming, thermostable α-amylase from *Pseudomonas saccharophila* (PSA).[18,20] Next to its exoaction that releases maltotetraose, this enzyme also shows endoaction. PSA has an intermediate DMA,[17] and it results in a faster decrease in starch molecular weight than does BStA.[18] Although the impact of BStA, BSuA, and PSA in starch model systems has been well studied, it has, to the best of our knowledge, not been clarified how these enzymes influence the starch network on mesoscale and, as a consequence, the texture properties of a real food system.

Against this background, the objective of this study was to link molecular changes in the starch fraction in bread to its mesoscale network organization and crumb texture properties. To that end, BStA, PSA, and BSuA, three α-amylases with different working mechanism and DMA, were added to a bread recipe, and low-resolution proton nuclear magnetic resonance (LR ^{1}H NMR) was used besides differential scanning calorimetry (DSC) and texture analyses to monitor their impact on starch network structures, molecular water distribution, and changes in crumb texture properties during storage.

MATERIALS AND METHODS

Materials and Analysis of Their Composition. Wheat flour (Surbi, milling yield of 75%) [71.5% starch, 11.1% protein, 13.9% moisture content (MC)] was provided by Dossche Mills (Deinze, Belgium). Soluble starch, that is, acid hydrolyzed potato starch, was from Merck (Darmstadt, Germany). BSuA was from Sigma-Aldrich (Bornem, Belgium). Novamyl and Powerflex, the sources of BStA and a mutant of PSA, respectively, were from Novozymes (Bagsvaerd, Denmark) and Danisco (Copenhagen, Denmark), respectively.

Starch content was calculated as 0.9 times the monosaccharide glucose content determined by gas−liquid chromatography as in Van Craeyveld et al.[21] Protein content was determined using an adaptation of the AOAC Official Method[22] 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 and bread crumb and crust was determined according to AACC method 44-15.02.[23] All reagents, solvents, and chemicals were of analytical grade and were obtained from Sigma-Aldrich unless indicated otherwise.

Amylase Activity Assay Based on Soluble Starch. Amylase activity was measured at 40 °C by quantifying the release of reducing sugars from 1.0% soluble starch in sodium maleate buffer (100 mM, pH 6.0) containing 5.0 mM $CaCl_2$ based on the assay outlined in Derde et al.[18] and Nelson.[24] BStA and PSA were extracted by suspending 50 mg of Novamyl and Powerflex, respectively, in 10 mL of the above sodium maleate buffer, shaking for 15 min, and separating the supernatant from the carrier by centrifugation (7800*g*, 10 min, 4 °C). Amylase solutions were then appropriately diluted in sodium maleate buffer and preincubated for 5 min at 40 °C. After the preincubation step, 500 μL of 1.0% soluble starch solution was added to the enzyme solutions (200 μL). At several time points (from 0 to 15 min), the reaction was stopped with an alkaline copper solution (500 μL),[24] and the liberated reducing end groups were quantified colorimetrically at 520 nm using a standard curve of maltose in deionized water (0−1.0 μmol/mL). One enzyme unit (EU) is the amount of enzyme releasing 1.0 μmol of maltose equivalents per mL from 1.0% soluble starch per minute at pH 6.0 and 40 °C. Measurements were performed in duplicate.

Bread Making. Control bread was made according to a straight-dough method.[25] 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. When enzyme (BStA, BSuA, or PSA) was used, it was dissolved in part of the dough water. A BStA dosage of 5.8 EU/g of flour (dry matter, DM) was used, which corresponds to earlier recommended dosages for bread making.[13] Dosages of BSuA and PSA were 0.17 EU/g DM flour and 5.9 EU/g DM flour, respectively. These dosages were chosen as they result in Rapid Visco Analysis (RVA) flour−water peak viscosities similar to those obtained when using the recommended dosage of BStA.[26] The dough was 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). Subsequent to molding and proofing (36 min at 30 °C, 90% relative humidity), dough was baked at 215 °C for 24 min in a National Manufacturing rotary hearth oven. Prior to further analyses, bread loaves were cooled for 2 h, and their volume was determined with a Volscan Profiler (Stable Micro Systems, Godalming, Surrey, UK). Bread loaves produced with BStA, PSA, or BSuA addition to their recipe are further referred to as BStA, PSA, or BSuA supplemented bread, respectively.

Storage of Bread and Crumb Sampling. After being cooled, three samples, each from the center of the crumb of a different bread (each approximately 0.3 g, accurately weighed), were placed in separate glass tubes, which were then sealed to prevent moisture loss. The tubes were stored at 25 °C and analyzed with LR ^{1}H NMR after storage for 2, 48, 120, and 168 h. In this way, the effect of the extent of amylopectin retrogradation on the proton distributions in bread crumb during storage was studied without interference of migration of water from crumb to crust. At the same time, fresh whole bread was wrapped in plastic foil and stored at 25 °C for 168 h in sealed plastic bags to prevent moisture loss. Center crumb samples were then also withdrawn and transferred into NMR tubes, which were then sealed. The additional effect of water migration from crumb to crust on the proton distributions during storage could now be studied. The bread loaves (three for each storage time) were further analyzed with DSC, crumb compression experiments, and ^{1}H NMR after storage for 2, 48, 120, and 168 h (see below).

Reducing Sugars Analysis. The concentration of reducing sugars in bread crumb was determined on the basis of Nelson.[24] Reducing sugars were extracted by suspending 50 mg of freeze-dried bread crumb in 1.0 mL of deionized water followed by shaking for 20 min at 6 °C. These mixtures were centrifuged (7500*g*, 10 min, 25 °C) and further appropriately diluted. Alkaline copper solution (500 μL) was added to the supernatant (700 μL). The liberated reducing end groups were quantified colorimetrically in duplicate as described for the determination of amylase activity.

Hydrolysis Products Analysis. The supernatant obtained after centrifugation for analysis of reducing sugars was diluted and analyzed in duplicate by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS 3000 chromatography system (Sunnyvale, CA), based on the assay outlined in Pollet et al.[27] with slight modifications. Samples were filtered (0.22 μm) and injected (25 μL) on Carbopac PA-100 guard and PA-100 anion exchange (250 × 4 mm) columns. Elution was with a linear gradient of 0−125 mM sodium acetate in 100 mM sodium hydroxide for 30 min (1.0 mL/min). Malto-oligosaccharide standards (glucose to maltoheptaose) and an internal standard (fucose) were used to identify and quantify the water extractable malto-oligosaccharides.

Differential Scanning Calorimetry. DSC was performed with a Q1000 DSC (TA Instruments, New Castle, DE). Bread crumb was freeze-dried after different storage times for analysis of retrograded amylopectin. Triplicate freeze-dried samples were accurately weighed (2.5−4.0 mg) in aluminum pans (Perkin-Elmer, Waltham, MA). Deionized water was added in a ratio of 1:3 (w/w, sample DM:water). The pans were hermetically sealed and equilibrated at 0 °C before heating to 120 °C at 4 °C/min (together with an empty reference pan). The system was calibrated with indium. The temperatures and enthalpies of the melting of amylopectin crystals formed as a result of retrogradation were determined using TA Instruments Universal Analysis software. Enthalpies were expressed in J/g sample (on DM basis).

We also determined the amount of freezable water (FW) in bread crumb. Hereto, 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

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

where $\Delta H_{\mathrm{melting}}$ is the melting enthalpy of ice in the sample (J/g sample), ΔH_{ice} is the melting enthalpy of ice (334 J/g ice), and MC is expressed in g water/g sample.[28]

The FW content of fresh bread crumb was analyzed in triplicate using samples withdrawn within 12 h after baking. For analysis after 48, 120, and 168 h, pans were filled with fresh crumb withdrawn after baking and stored at 25 °C.

Crumb Texture Analysis. Firmness, resilience, and stiffness of bread crumb were measured as a function of storage time by compression with an Instron 3342 (Norwood, MA). Five cylindrical samples (height 25 mm; diameter 30 mm) were cut with a sharp borer from the center of three different fresh and stored bread loaves. A cylindrical probe (diameter 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. Resilience was determined as the percent recoverable work in a compression−decompression cycle with 30% compression. The percent recoverable work was calculated from the areas under the compression and decompression curves as

$$\%\text{recoverable work} = \frac{\text{area under decompression curve}}{\text{area under compression curve}} \times 100$$

in a stress−strain curve.[29−31] Crumb stiffness was determined as Young's modulus, that is, the ratio of stress to strain when deformation is totally elastic.[30] To take loaf density into account, the corrected Young's modulus was calculated as

$$\text{corrected Young's modulus} = \frac{\text{Young's modulus}}{\text{density}^2}$$

where Young's modulus is expressed in Pa, density is in kg/m^3, and therefore corrected Young's modulus is in Pa/(kg/m^3)^2.[32]

Low-Resolution Proton Nuclear Magnetic Resonance. Measurements of proton distributions in bread crumb 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. Spin−spin relaxation times (T_2) were determined. The relaxation curves were acquired using a single 90° pulse (free induction decay, FID)[33] and a Carr−Purcell−Meiboom−Gill (CPMG) pulse sequence.[34] 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.

Three samples of crumb (each approximately 0.3 g, accurately weighed) were placed in three different Bruker NMR tubes (10 mm external diameter) and tightly compressed to a height of 8 mm to remove air bubbles. The tubes were sealed and analyzed in triplicate. The exact sample weight in each tube was taken into account in the computations.

The CONTIN algorithm of Provencher[35] (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 area under the curve of a population with a certain T_2 is proportional to the number of protons in that population. Because the inhomogeneity of the static magnetic field affected the output for the most mobile FID population (around 0.5 ms), this fraction was not taken into account in the following analyses.

Statistical Analysis. Statistical analyses were conducted with the Statistical Analysis System software 9.2 (SAS Institute, Cary, NC). For several variables, it was verified whether mean values, based on at least three individual measurements, were significantly different at a significance level (α) of 0.05 using the two-way ANOVA procedure.

RESULTS AND DISCUSSION

Amylase Action on Starch Molecules during Bread Baking and Storage. Addition of the different enzymes to the bread making recipe increased the levels of reducing saccharides in fresh bread crumb (Figure 1), showing that all amylases had been active during baking. The substantial concentration of reducing saccharides in control bread crumb can be explained by the activity of endogenous α- and β-amylases and by the presence of reducing sugars formed by yeast invertase from the sucrose in the recipe. After storage for 168 h, no clear change in the level of reducing ends was observed for the control bread (Figure 1). BStA hydrolyzed starch to form mainly malto-oligosaccharides with a degree of polymerization (DP) of 2−4, but also small levels of larger malto-oligosaccharides (DP 5−7) (Figure 2a). Apparently, this amylase was still active after baking. Indeed, the level of maltose (DP 2), and thus of reducing ends, further increased during storage for 168 h (Figures 2b and 1, respectively). PSA mainly hydrolyzed starch with release of maltotetraose (DP 4), but also small levels of maltose and maltohexaose (DP 6) were formed (Figure 2a). The level of the different malto-oligosaccharides did not increase during storage (Figures 1 and 2b), showing that PSA was no longer active after baking. The main saccharide hydrolysis products found in BSuA supplemented bread were maltohexaose and maltopentaose (DP 7). The amylase also formed maltose (Figure 2a). During storage of BSuA supplemented bread for 168 h, the concentration of reducing ends increased with 46% (Figure 1), showing that this enzyme was still active after baking with additional formation of malto-oligosaccharides of DP 2−7 during storage (Figure 2b). The activity of BStA and BSuA remaining after baking was in line with earlier findings.[19]

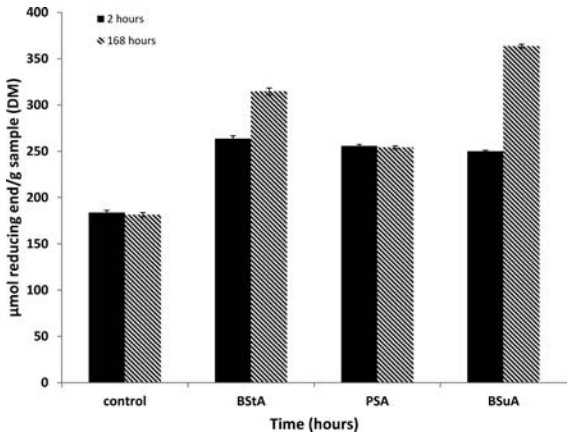

Figure 1. Reducing ends [μmol/g of crumb (dry matter, DM)] from control bread (no added amylase) and bread supplemented with *Bacillus stearothermophilus* α-amylase (BStA), *Pseudomonas saccharophila* α-amylase (PSA), or *Bacillus subtilis* α-amylase (BSuA) after 2 and 168 h of storage at 25 °C. Measurements were performed in duplicate.

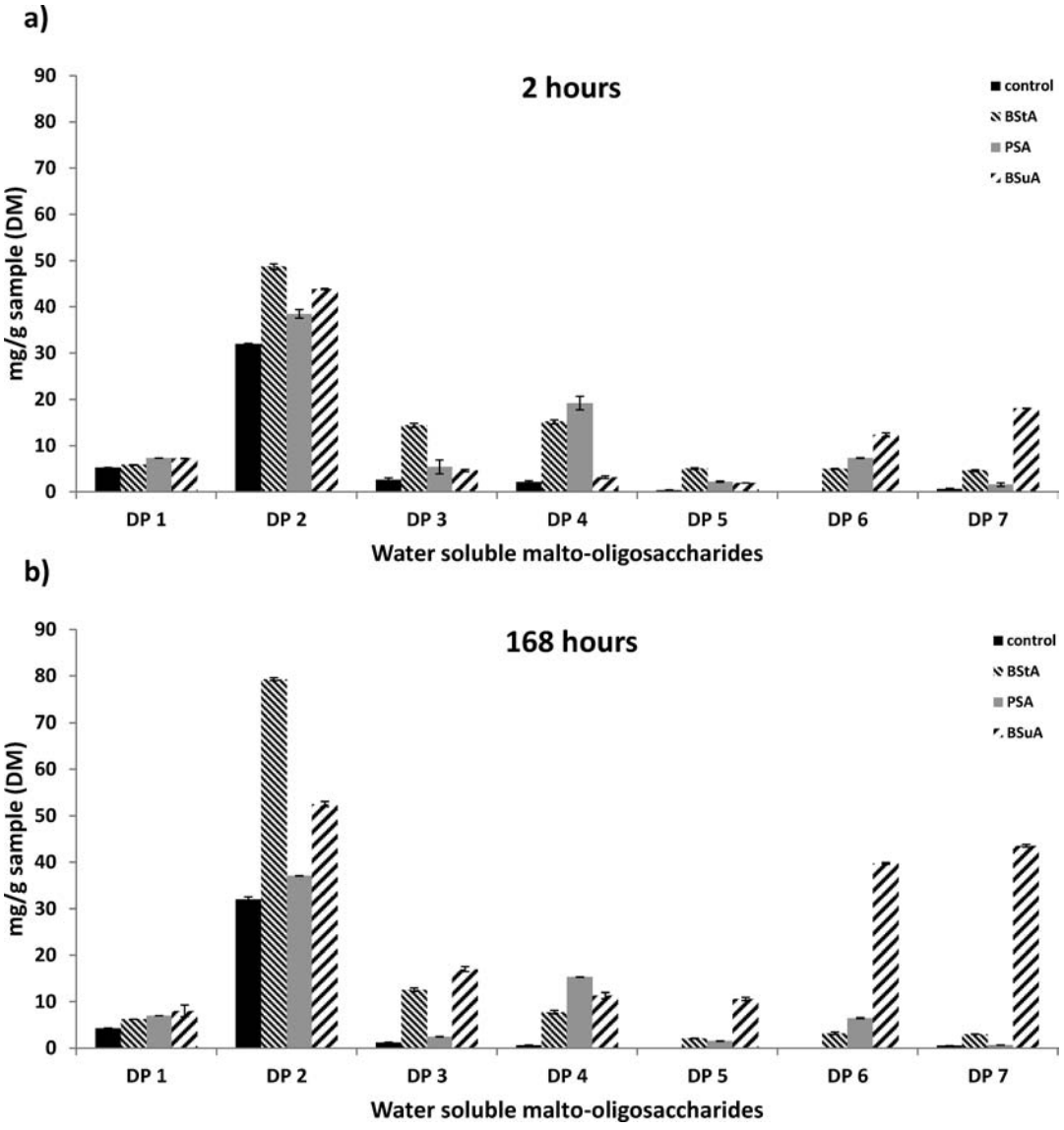

Figure 2. Linear malto-oligosaccharides with a degree of polymerization (DP) 1−7 present in fresh crumb [expressed on dry matter (DM) basis] after 2 h (a) and crumb stored for 168 h at 25 °C (b) from control bread and bread supplemented with *Bacillus stearothermophilus* α-amylase (BStA), *Pseudomonas saccharophila* α-amylase (PSA), and *Bacillus subtilis* α-amylase (BSuA). Measurements were performed in duplicate.

Physical Changes of Bread Crumb during Storage. The MC of the center crumb decreased and crust MC increased in a similar way for all bread loaves during storage for 168 h at 25 °C. Toward the end of the storage period, moisture migration from crumb to crust leveled off (results not shown). The volumes of bread loaves from 100 g of flour were significantly higher ($P < 0.05$) for loaves supplemented with BStA (651 cm^3) and PSA (685 cm^3) than for those of control bread (632 cm^3) and bread loaves supplemented with BSuA (637 cm^3). Amylase action affects dough viscosity during oven rise,[7] and this presumably impacted bread volume when BStA or PSA was used. The bread volume enhancing effect of PSA has been described earlier.[36] In the particular case of BSuA use, it seems probable that the experimental dosage had little impact on dough viscosity.

Despite partial hydrolysis of the starch fraction by amylases during baking (Figures 1 and 2a), no significant differences with control bread in terms of initial crumb firmness and stiffness were observed (Table 1). For amylase supplemented bread loaves, the initial crumb resilience and its decrease during storage were lower than for control bread (Table 1). While for bread supplemented with BSuA a minimum resilience was reached after 48 h, resilience was better preserved for bread loaves supplemented with BStA, and, to a lesser extent those supplemented with PSA, than for control loaves (Table 1). Irrespective of its source, amylase addition reduced the firmness of the crumb in such a way that, after storage for 168 h, it was significantly lower than that of control bread, with BStA and BSuA resulting in the softest bread crumb (Table 1). In the case of PSA addition, the smaller differences in firmness and resilience than those of control bread were mainly caused by the higher volume of these bread loaves, because Young's modulus when corrected for density was not significantly different from that of control bread stored for 168 h (Table 1). The portion of FW was initially similar for all bread loaves but decreased significantly during storage for control bread and bread supplemented with PSA, while no significant changes in FW were observed for bread supplemented with BStA. Remarkably, FW of bread supplemented with BSuA initially increased and then decreased (Table

Table 1. Crumb Firmness,[a] Crumb Resilience,[a] Corrected Young's Modulus,[a] Freezable Water (FW),[b] and Melting Enthalpy of Retrograded Amylopectin (ΔH_{AP})[b] of Crumb from Control Bread (No Added Amylase) and Bread Supplemented with *Bacillus stearothermophilus* α-Amylase (BStA), *Pseudomonas saccharophila* α-Amylase (PSA), or *Bacillus subtilis* α-Amylase (BSuA) Stored for 168 h at 25 °C[c]

additive	storage time (h)	crumb firmness (N)	crumb resilience (%)	corrected Young's modulus [Pa/(kg/m^3)^2]	FW (%)	ΔH_{AP} [J/g crumb (DM)]
none	2	1.1 (0.2)	54.0 (2.1)	0.2 (0.0)	47.0 (4.1)	0.25 (0.02)
	48	2.4 (0.1)	45.9 (1.5)	0.4 (0.0)	48.0 (1.3)	2.03 (0.06)
	120	4.8 (0.2)	34.2 (1.3)	1.0 (0.0)	39.9 (4.5)	3.26 (0.18)
	168	7.0 (0.5)	30.3 (0.4)	1.6 (0.1)	38.5 (1.7)	3.34 (0.22)
BStA	2	1.1 (0.1)	40.4 (2.0)	0.2 (0.0)	51.4 (1.5)	0.15 (0.06)
	48	1.5 (0.1)	39.1 (0.8)	0.3 (0.0)	47.3 (4.1)	0.43 (0.03)
	120	2.2 (0.2)	36.8 (1.3)	0.5 (0.0)	48.9 (0.3)	0.63 (0.06)
	168	2.4 (0.1)	35.5 (0.9)	0.5 (0.1)	49.7 (2.1)	0.80 (0.11)
PSA	2	1.0 (0.2)	47.3 (1.3)	0.2 (0.0)	52.8 (1.5)	0.28 (0.05)
	48	2.4 (0.3)	42.6 (0.8)	0.6 (0.1)	47.6 (2.3)	1.02 (0.07)
	120	3.7 (0.5)	37.6 (1.8)	1.1 (0.1)	45.8 (3.3)	2.00 (0.26)
	168	5.7 (0.2)	34.8 (0.8)	1.8 (0.1)	44.1 (2.0)	2.24 (0.08)
BSuA	2	1.2 (0.2)	22.9 (2.2)	0.1 (0.0)	50.9 (2.4)	0.37 (0.08)
	48	1.5 (0.1)	12.7 (0.6)	0.2 (0.0)	55.4 (1.3)	1.26 (0.09)
	120	1.6 (0.2)	13.5 (1.1)	0.3 (0.0)	54.0 (1.0)	1.54 (0.20)
	168	1.8 (0.2)	13.7 (1.8)	0.3 (0.1)	52.0 (2.9)	1.55 (0.02)

[a]Number of samples = 5. [b]Number of samples = 3. [c]Standard deviations are given between brackets.

Table 2. Relaxation Time (T_2) and Area of the First Free Induction Decay (FID) Population A (Rigid CH Protons of Starch Crystals and Amorphous Starch and Gluten Not in Contact with Water), FID Population B (Mobile CH Protons from Amorphous Starch and Gluten in Little Contact with Water), Carr−Purcell−Meiboom−Gill (CPMG) Population C (Mobile CH Protons from Amorphous Starch and Gluten in Little Contact with Water), and CPMG Population E (Mobile Exchanging Protons of Water, Starch, and Gluten in the Formed Gel Network) of Crumb during 168 h of Storage at 25 °C in NMR Tubes (No Crumb to Crust Moisture Migration) from Control Bread (No Added Amylase) and Bread Supplemented with *Bacillus stearothermophilus* α-Amylase (BStA), *Pseudomoas saccharophila* α-Amylase (PSA), or *Bacillus subtilis* α-Amylase (BSuA)[a]

		population A (FID)		population B (FID)		population C (CPMG)		population E (CPMG)	
additive	storage time (h)	T_2 (μs)	area (au)	T_2 (μs)	area (au)	T_2 (ms)	area (au)	T_2 (ms)	area (au)
none	2	14 (2)	4885 (462)	63 (6)	1015 (323)	0.32 (0.01)	1258 (96)	8.30 (0.18)	8961 (34)
	48	14 (0)	6884 (378)	67 (6)	726 (54)	0.37 (0.06)	872 (147)	7.92 (0.07)	8960 (31)
	120	14 (0)	7611 (28)	73 (6)	637 (54)	0.41 (0.07)	1111 (244)	7.60 (0.03)	9044 (25)
	168	13 (1)	8555 (461)	63 (6)	751 (37)	0.40 (0.03)	1066 (210)	7.56 (0.16)	8978 (77)
BStA	2	14 (0)	5356 (123)	60 (0)	549 (35)	0.36 (0.03)	968 (68)	8.87 (0.12)	9102 (44)
	48	14 (0)	6081 (205)	57 (6)	572 (50)	0.31 (0.01)	1032 (20)	8.77 (0.12)	9164 (27)
	120	14 (1)	6018 (51)	63 (6)	377 (59)	0.33 (0.06)	719 (82)	8.83 (0.06)	9198 (67)
	168	14 (0)	6177 (63)	63 (6)	384 (73)	0.31 (0.01)	807 (137)	8.93 (0.15)	9177 (47)
PSA	2	14 (1)	5430 (233)	63 (6)	538 (17)	0.31 (0.01)	976 (37)	8.53 (0.06)	9133 (109)
	48	14 (1)	6170 (53)	57 (6)	490 (39)	0.37 (0.06)	733 (10)	8.21 (0.12)	9302 (61)
	120	13 (1)	7004 (258)	60 (0)	412 (64)	0.33 (0.06)	849 (66)	8.15 (0.05)	9352 (56)
	168	14 (0)	7127 (105)	77 (21)	239 (79)	0.33 (0.06)	601 (244)	7.99 (0.35)	9294 (126)
BSuA	2	14 (1)	6328 (477)	57 (6)	430 (51)	0.31 (0.01)	1010 (159)	8.20 (0.10)	9073 (74)
	48	14 (0)	7231 (263)	100 (0)	131 (22)	0.38 (0.03)	780 (160)	8.37 (0.12)	9494 (82)
	120	13 (0)	7575 (148)	73 (23)	118 (39)	0.33 (0.06)	525 (62)	8.80 (0.10)	9655 (46)
	168	14 (0)	7348 (39)	100 (0)	63 (6)	0.33 (0.06)	441 (285)	8.93 (0.15)	9738 (42)

[a]T_2's are given in μs (populations A and B) or ms (populations C and E), and areas are given in arbitrary units (au). Standard deviations are given between brackets. The average of three measurements obtained with crumb from three different bread loaves was taken for each storage time.

1). Bread supplemented with BStA and BSuA had significantly higher end values for FW than control bread after storage.

To further understand the differences in firmness, resilience, and FW (physical changes) during storage of bread supplemented with different amylases, amylopectin crystal formation (DSC) and proton distributions (^{1}H NMR measurements) (molecular changes) were determined.

Changes in Biopolymer Networks and Water Distribution during Bread Storage. For the NMR experiments, only crumb samples of the different bread loaves stored in sealed NMR tubes are discussed, because crumb to crust water migration did not differ between the studied bread loaves (see section Physical Changes of Bread Crumb during Storage). In the crumb of fresh bread, the area of ^{1}H NMR population A, which contains rigid CH protons of amylose crystals and amorphous starch and gluten not in contact with water,[37] was higher for bread supplemented with amylases than for control bread (Table 2). In addition, the areas of populations B and C, both containing mobile CH protons from amorphous starch and gluten in little contact with water,[37] were initially lower in

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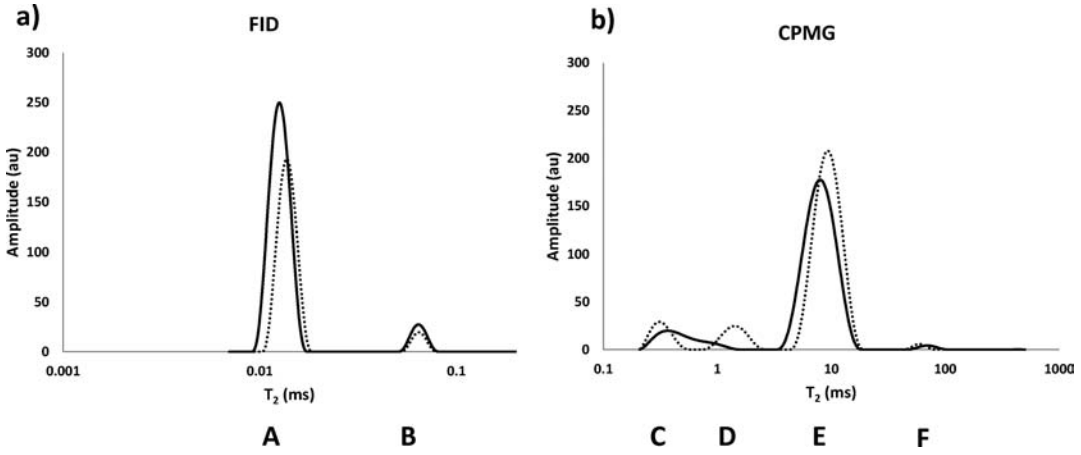

Figure 3. Free induction decay (FID) (a) and Carr−Purcell−Meiboom−Gill (CPMG) (b) proton distributions of crumb removed after 2 h of cooling and stored for 168 h in LR ^{1}H NMR tubes from control bread (−) and from bread supplemented with *Bacillus stearothermophilus* α-amylase (···). The distributions were 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. Measurements were performed on crumb from three different bread loaves for each storage time.

amylase treated bread (Table 2). These differences show that amylase addition to the recipes resulted in the presence of more rigid CH protons in the crumb of fresh, cooled bread at the expense of mobile CH protons. Possibly, the increased mobility of amylose after hydrolysis by BStA, PSA, and especially BSuA promoted formation of rigid amylose crystals during bread cooling. The higher amount of amylose crystals was reflected in the lower initial resilience of bread supplemented with amylase than that of control bread (Table 1). The enhanced formation of amylose crystals observed with ^{1}H NMR and texture analyses confirms earlier hypotheses about fast formation of an amylose network during cooling that results in texture stabilization[6,13,19] due to the presence of a more rigid amylose network with prevention of structure collapse.

Melting enthalpy of retrograded amylopectin in fresh crumb was similar for all bread loaves, but the value after storage for 168 h was significantly lower for amylase supplemented bread than for control bread (Table 1).

Especially for bread supplemented with BStA, the increase in melting enthalpy was very low (Table 1). The limited retrogradation was also observed with ^{1}H NMR as a smaller increase in area of population A and smaller decreases in areas of populations B and C than for crumb of control bread (Figure 3 and Table 2). Hence, BStA did not only enhance amylose crystallization initially, but also prevented amylopectin retrogradation during storage (Table 1). The prevention of amylopectin crystal formation by use of maltogenic α-amylase has also been reported by others.[6,13] The lack of a decrease in mobility (T_2) of population E, which contains mobile exchanging protons of water, starch, and gluten in the formed gel network[37] (Figure 3b and Table 2), and only a small decrease in FW (Table 1) during storage of BStA containing bread pointed to only little amylopectin network formation during storage. Less amylopectin crystals resulted in less crystalline junction zones and, therefore, in a less rigid, continuous, crystalline starch network during storage with inclusion of water.[8] Also, populations C and D (the latter containing CH protons of gluten and exchanging protons of confined water, starch and gluten[37]) did not merge during storage in bread supplemented with BStA in the way they did for control bread (Figure 3b). The well-separated populations C and D (Figure 3b) in BStA supplemented bread indicated a less extended starch network without water inclusion, resulting in a better plasticized gluten network. For control bread, the reduced amount of water associated with the gluten strands caused merging of populations C and D (Figure 3b). This reduction in gluten-associated water was caused by diffusion of water to the starch due to the thermodynamic immiscibility of starch and gluten[8,12] and by inclusion of water into the formed starch network during storage, the latter leading to a decrease in FW.[8] The lack of extended starch network formation and the conservation of gluten plasticization in BStA supplemented bread were responsible for the small increase in firmness and stiffness and in the nearly unaffected resilience during storage (Table 1).

Addition of PSA also resulted in bread with less amylopectin retrogradation and, therefore, a smaller increase in area of population A than in that of control bread crumb (Table 2). However, the changes were less pronounced than for BStA supplemented bread. The higher tendency of PSA to perform endoaction[17] and the fact that it was no longer active during storage (Figures 1 and 2) resulted in amylopectin molecules that were still able to retrograde (Table 1) and still allowed formation of a more extended starch network that included more water than that in bread supplemented with BStA. This was verified by a larger decrease in mobility (T_2) of population E (Table 2) and a larger decrease in FW than for bread supplemented with BStA (Table 1). Also, populations C and D merged during storage of crumb in sealed NMR tubes (results not shown). This pointed to a reduction in the quantity of water associated with the gluten strands.[8] On the basis of the results obtained for bread supplemented with PSA, it can be concluded that the main effect of the enzyme was a higher loaf volume and a lower initial crumb resilience due to enhanced amylose crystallization.

For bread supplemented with BSuA, the melting enthalpy of retrograded amylopectin was significantly higher after 48 h of storage, but leveled off after 120 h of storage and was lower after 168 h than that in the case of PSA supplemented bread (Table 1). BSuA not only formed large oligosaccharides during bread storage, but also fragments of DP 2−4 (Figure 2b). We speculate that amylopectin initially was still capable of forming crystals, but that the continuing activity of BSuA but not of PSA during storage (Figures 1 and 2b) resulted in further hydrolysis of the

remaining amorphous amylopectin chains, which were therefore prevented from retrograding. During storage, the areas of populations B and C in bread produced with BSuA addition decreased to a larger extent than those observed for the other bread loaves during storage (Table 2). Amylopectin retrogradation led to a shift of (part of) the amorphous CH protons of amylopectin that organized themselves into crystalline structures to lower mobility, shown as an increased area of the rigid population A.[8] Additionally, due to the further hydrolysis of starch by BSuA during storage, the mobility of (part of) the amorphous starch CH protons increased, shown as a shift to the more mobile population E (Table 2). The hydrolysis of the amorphous chains linking the different crystalline regions or micelles during storage also released water. This was observed as a higher portion of FW than for control bread (Table 1). The more mobile water protons also contributed to the increased area of population E during storage (Table 2). Besides the higher amount of amylose crystals in fresh bread crumb, shown as a high initial area of ^{1}H NMR population A (Table 2), it can be hypothesized that during the first 48 h of storage mostly the chains in the amylose network were hydrolyzed, because the amount of FW after 48 h was significantly higher than for control bread. This resulted in a very low resilience and an increased crumb stickiness after 48 h of storage due to partial structure collapse. Mouneim et al.[38] also found a higher amount of FW in bread when bacterial α-amylase had been added to its recipe. The overall loss of rigidity of the starch network was observed as an increased mobility (T_2) of population E during storage (Table 2). The softer starch network and the release of included water prevented the increase in crumb firmness due to amylopectin retrogradation (Table 1). The gluten network remained plasticized, because no complete merging of populations C and D during storage of crumb was observed with ^{1}H NMR (results not shown). The absence of changes in amylopectin retrogradation after 120 h of storage and the full gluten plasticization can explain the absence of substantial changes in crumb firmness, resilience, stiffness, and FW after 120 h in bread supplemented with BSuA (Table 1).

Earlier, our group showed that the strength of the starch gel network is related to T_2 of population E in bread crumb. During the first days of bread storage, amylopectin retrogradation largely impacted crumb firmness, while stiffening of the gluten network due to loss of plasticizing water (inter alia caused by crumb to crust moisture migration) contributed to crumb firmness after a couple of days of storage. To include the impact of crumb to crust moisture migration on crumb firmness, we studied the relationship between firmness and T_2 of population E from samples collected from intact bread loaves.[8] For control bread and bread supplemented with BStA or PSA, a negative linear relation was found between firmness and T_2 of population E during storage of bread as a whole (Figure 4). For bread supplemented with BSuA, no relation could be found, because T_2 of population E was additionally impacted by the weakening of the starch network. A similar slope was obtained for bread supplemented with PSA and control bread, which indicates that the overall rigidities of the amylopectin network formed during storage were comparable in both bread types. The similar decrease in FW confirms this hypothesis. For the bread supplemented with BStA, a steeper slope was seen than for control bread or bread supplemented with PSA (Figure 4). For the same decrease in T_2 of population E, the increase in firmness was smaller. This confirms that BStA led to formation of a less extended starch network during storage.

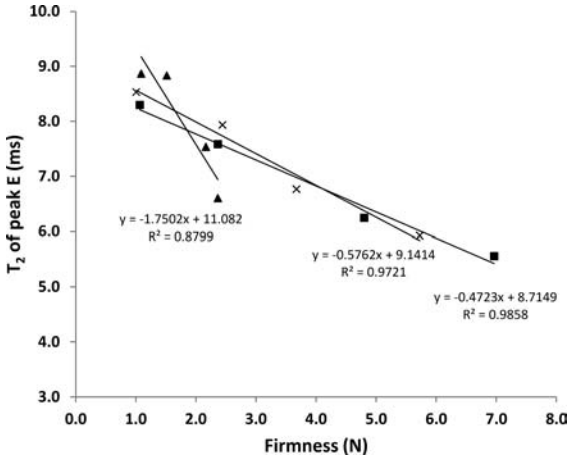

Figure 4. The relaxation time (T_2) of the most mobile Carr−Purcell−Meiboom−Gill (CPMG) population E, representing mobile exchanging protons of water, starch, and gluten present in the formed gel network (measured with proton nuclear magnetic resonance), and firmness (measured with texture analysis) are linearly related for crumb from control bread (■, R^2 = 0.9858), for crumb from bread supplemented with *Bacillus stearothermophilus* α-amylase (▲, R^2 = 0.8799), and for crumb from bread supplemented with *Pseudomonas saccharophila* α-amylase (×, R^2 = 0.9721). Bread loaves were stored with crust for 168 h. The average of three (LR ^{1}H NMR) or five (texture analyses) measurements obtained with crumb from three different bread loaves was taken for each storage time.

BStA, PSA, and BSuA reduced the firmness of control bread. Higher crumb softness was caused by increased loaf volume (PSA) and by starch degradation (BStA, PSA, and BSuA). The extent of starch degradation was of importance, because a too high degree of degradation resulted in partial structure collapse and sticky bread crumbs as was the case with BSuA. In general, an efficient antifirming amylase increases bread volume and prevents amylopectin retrogradation, and thus formation and reinforcement of an extended starch network, but does not result in crumb structure collapse. Of the three amylases used in this study, BStA proved to be the most efficient antifirming amylase. The exomode of action and the high DMA of this enzyme resulted in a more rigid initial amylose network and in hydrolysis of the amylopectin side chains. The latter action is responsible for its antifirming properties.

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Notes

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ABBREVIATIONS USED

BStA, *Bacillus stearothermophilus* maltogenic α-amylase; BSuA, *Bacillus subtilis* α-amylase; DMA, degree of multiple attack; PSA, *Pseudomonas saccharophila* α-amylase; LR ^{1}H NMR, low-resolution proton nuclear magnetic resonance; DSC, differential scanning calorimetry; MC, moisture content; EU, enzyme unit; DM, dry matter; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; FW, freezable water; $\Delta H_{melting}$, melting enthalpy of ice in the sample; ΔH_{ice}, melting enthalpy of ice; T_2, spin−spin relaxation time; FID, free induction decay; CPMG, Carr−Purcell−Meiboom−Gill; DP, degree of polymerization; au, arbitrary units; ΔH_{AP}, melting enthalpy of retrograded amylopectin.

REFERENCES

(1) Willhoft, E. M. Bread staling. 2. Theoretical study. *J. Sci. Food Agric.* **1971**, *22*, 180−183.

(2) Kim, S. K.; D'Appolonia, B. L. Bread staling studies.II. Effect of protein content and storage temperature on the role of starch. *Cereal Chem.* **1977**, *54*, 216−224.

(3) Ribotta, P. D.; Le Bail, A. Thermo-physical assessment of bread during staling. *Lebensm.-Wiss. Technol.* **2007**, *40*, 879−884.

(4) Lagrain, B.; Wilderjans, E.; Glorieux, C.; Delcour, J. A. Importance of gluten and starch for structural and textural properties of crumb from fresh and stored bread. *Food Biophys.* **2012**, *7*, 173−181.

(5) Gray, J. A.; Bemiller, J. N. Bread staling: Molecular basis and control. *Compr. Rev. Food Sci. Food Saf.* **2003**, *2*, 1−21.

(6) Hug-Iten, S.; Escher, F.; Conde-Petit, B. Staling of bread: role of amylose and amylopectin and influence of starch-degrading enzymes. *Cereal Chem.* **2003**, *80*, 654−661.

(7) Goesaert, H.; Slade, L.; Levine, H.; Delcour, J. A. Amylases and bread firming: an integrated view. *J. Cereal Sci.* **2009**, *50*, 345−352.

(8) Bosmans, G. M.; Lagrain, B.; Ooms, N.; Fierens, E.; Delcour, J. A. Biopolymer interactions, water dynamics and bread crumb firming. *J. Agric. Food Chem.* **2013**, *61*, 4646−4654.

(9) Slade, L.; Levine, H. Non-equilibrium melting of native granular starch: Part I. Temperature location of the glass transition associated with gelatinization of A-type cereal starches. *Carbohydr. Polym.* **1988**, *8*, 183−208.

(10) Slade, L.; Levine, H. Beyond water activity: recent advances based on an alternative approach to the assessment of food quality and safety. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*, 115−360.

(11) Eerlingen, R. C.; Deceuninck, M.; Delcour, J. A. Enzyme-resistant starch. II. Influence of amylose chain length on resistant starch formation. *Cereal Chem.* **1993**, *70*, 345−350.

(12) Tolstoguzov, V. Thermodynamic aspects of dough formation and functionality. *Food Hydrocolloids* **1997**, *11*, 181−193.

(13) Goesaert, H.; Leman, P.; Bijttebier, A.; Delcour, J. A. Antifirming effects of starch degrading enzymes in bread crumb. *J. Agric. Food Chem.* **2009**, *57*, 2346−2355.

(14) Gerrard, J. A.; Every, D.; Sutton, K. H.; Gilpin, M. J. The role of maltodextrins in the staling of bread. *J. Cereal Sci.* **1997**, *26*, 201−209.

(15) Bijttebier, A.; Goesaert, H.; Delcour, J. A. Temperature impacts the multiple attack action of amylases. *Biomacromolecules* **2007**, *8*, 765−772.

(16) Robyt, J. F.; French, D. Multiple attack hypothesis of alpha-amylase action: action of porcine pancreatic, human salivary and *Aspergillus oryzae* alpha-amylases. *Arch. Biochem. Biophys.* **1967**, *122*, 8−16.

(17) Derde, L. J.; Gomand, S. V.; Courtin, C. M.; Delcour, J. A. Hydrolysis of β-limit dextrins by α-amylases from porcine pancreas, Bacillus subtilis, Pseudomonas saccharophila and Bacillus stearothermophilus. *Food Hydrocolloids* **2012**, *26*, 231−239.

(18) Derde, L. J.; Gomand, S. V.; Courtin, C. M.; Delcour, J. A. Characterisation of three starch degrading enzymes: Thermostable β-amylase, maltotetraogenic and maltogenic α-amylases. *Food Chem.* **2012**, *135*, 713−721.

(19) Lagrain, B.; Leman, P.; Goesaert, H.; Delcour, J. A. Impact of thermostable amylases during bread making on wheat bread crumb structure and texture. *Food Res. Int.* **2008**, *41*, 819−827.

(20) Kragh, K. M.; Larsen, B.; Rasmussen, P.; Duedahl-Olesen, L.; Zimmermann, W. Non-maltogenic exoamylases and their use in retarding retrogradation of starch. US 6890572 B2, 2005.

(21) Van Craeyveld, V.; Delcour, J. A.; Courtin, C. M. Extractability and chemical and enzymic degradation of psyllium (Plantago ovata Forsk) seed husk arabinoxylans. *Food Chem.* **2009**, *112*, 812−819.

(22) AOAC. *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Washington, DC, 1995.

(23) AACC. *Approved Methods of Analysis*, 11th ed.; Method 44-15.02. Moisture - Air-Oven Methods. Approved November 3, 1999; AACC International: St. Paul, MN, 1999; http://dx.doi.org/10.1094/AACCIntMethod-44-15.02.

(24) Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* **1944**, *153*, 375−380.

(25) Finney, K. F. An optimized, straight-dough, bread-making method after 44 years. *Cereal Chem.* **1984**, *61*, 20−27.

(26) Leman, P.; Goesaert, H.; Vandeputte, G. E.; Lagrain, B.; Delcour, J. A. Maltogenic amylase has a non-typical impact on the molecular and rheological properties of starch. *Carbohydr. Polym.* **2005**, *62*, 205−213.

(27) Pollet, A.; Beliën, T.; Fierens, K.; Delcour, J. A.; Courtin, C. M. Fusarium graminearum xylanases show different functional stabilities, substrate specificities and inhibition sensitivities. *Enzyme Microb. Technol.* **2009**, *44*, 189−195.

(28) Baier-Schenk, A.; Handschin, S.; Conde-Petit, B. Ice in prefermented frozen bread dough - An investigation based on calorimetry and microscopy. *Cereal Chem.* **2005**, *82*, 251−255.

(29) Kou, Y.; Chinachoti, P. Structural damage in bread staling as detected by recoverable work and stress-strain model analysis. *Cereal Foods World* **1991**, *36*, 888−892.

(30) Liu, Z.; Scanlon, M. G. Revisiting crumb texture evaluation methods: tension, compression and indentation. *Cereal Foods World* **2004**, *49*, 76−82.

(31) Nussinovitch, A.; Steffens, M. S.; Chinachoti, P. Elastic properties of bread crumb. *Cereal Chem.* **1992**, *69*, 678−681.

(32) Gibson, L. J.; Ashby, M. F. *Cellular Solids: Structure & Properties*; Cambridge University Press: New York, 1999; p 510.

(33) Ruan, R. R.; Chen, P. L. *Water in Foods and Biological Materials: A Nuclear Magnetic Resonance Approach*; Technomic Publishing Co., Inc.: PA, 1998; p 298.

(34) Meiboom, S.; Gill, D. Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instrum.* **1958**, *29*, 688−691.

(35) Provencher, S. W. Contin: a general purpose constrained regularization program for inverting noisy linear algebraic and integral equations. *Comput. Phys. Commun.* **1982**, *27*, 229−242.

(36) Kragh, K. M.; Kellet-Smith, A. H.; Shaw, A.; Chin, R. *Pseudomonas saccharophila* G4-amylase variants and uses thereof. WO 2009/083592 A1, 2009.

(37) Bosmans, G. M.; Lagrain, B.; Deleu, L. J.; Fierens, E.; Hills, B. P.; Delcour, J. A. Assignments of proton populations in dough and bread using NMR relaxometry of starch, gluten, and flour model systems. *J. Agric. Food Chem.* **2012**, *60*, 5461−5470.

(38) Mouneim, H.; Besbes, E.; Jury, V.; Monteau, J.-Y.; Le-Bail, A. Combined effects of baking conditions and bacterial α-amylases on staling kinetics of degassed and porous bread crumb. *Food Bioprocess Technol.* **2012**, *5*, 3032−3041.