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Gluten-Free Sorghum Bread Improved by Sourdough Fermentation: Biochemical, Rheological, and Microstructural Background

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This study was conducted to improve the quality and theoretical understanding of gluten-free sorghum bread. The addition of 2% hydroxypropyl methylcellulose improved bread based on 105% water, 70% sorghum flour, and 30% potato starch. Nevertheless, a flat top and tendency toward a hole in the crumb remained. Sourdough fermentation of the total sorghum flour eliminated these problems. Size-exclusion high-performance liquid chromatography demonstrated that during sourdough fermentation, proteins from the dough liquid were degraded to peptides smaller than kafirin monomers (<19 kDa). Laser scanning confocal microscopy showed aggregated protein in bread crumb without sourdough fermentation, whereas with sourdough fermentation, only small isolated patches of protein bodies embedded in matrix protein remained. In oscillatory temperature sweeps, sourdough fermentation caused a significantly higher resistance to deformation ($|G^*|$) after gelatinization of the above batter relative to batters without sourdough. Results suggest that a strong starch gel, without interference of aggregated protein, is desirable for this type of bread.

KEYWORDS: Sorghum; gluten-free bread; sourdough; size-exclusion-HPLC (SE-HPLC, SEC); rheology; laser scanning confocal microscopy (LSCM)

INTRODUCTION

Celiac disease is a syndrome characterized by damage to the mucosa of the small intestine caused by ingestion of certain wheat proteins and related proteins in rye and barley (*1*). While it has been shown decades ago that wheat gliadins are toxic to persons with celiac disease (*2*, *3*), more recent work has demonstrated the same for wheat glutenins (*4*, *5*). Sorghum [*Sorghum bicolor* (L.) Moench] is often recommended as a safe food for celiac patients, because it is more closely related to maize than to wheat, rye, and barley (*6*). It might, therefore, provide a good basis for gluten-free bread. However, the bulk of studies dealing with leavened breads containing sorghum have focused on composite breads from wheat and sorghum, in which a maximum of only 30% sorghum is regarded as acceptable (*7*). Such breads are not gluten-free and thus inappropriate for celiac patients.

Gluten-free breads in general require a different technology. The properties of the dough are more fluid than wheat dough and closer in viscosity to cake batters (*8*) due to the lack of a gluten network. Furthermore, gas holding is more difficult, and the use of gums (hydrocolloids), stabilizers, and pregelatinized starch has been suggested as a means to provide gas occlusion and stabilizing mechanisms (*8*, *9*). Specifically in the case of sorghum, the use of certain methylcellulose derivatives as hydrocolloid additives was found to be effective in improving gas retention and preventing collapse of the loaves during baking (*10*). Furthermore, the addition of pure starches caused a finer crumb (*10*). However, no explanation for the physicochemical effects of these substances was provided.

In contrast to general opinion, wheat-free sorghum bread may also be produced without any type of hydrocolloid, stabilizer, and pregelatinized starch, when about 30% raw starch is added to the sorghum flour (*11*). In a previous study (*12*), we focused on the latter type of bread and compared different sorghum hybrids. This bread was acceptable when fresh; however, its volume was small, and it tended to become unacceptably firm and brittle within 1 day of storage. For the present study, preliminary experiments showed that the use of hydroxypropyl methylcellulose (HPMC) not only improved the quality of fresh bread (*10*) but also delayed staling. In wheat bread, the same antistaling effect has been found and explained with water retention of HPMC and its tendency to bind to starch, thus possibly inhibiting amylopectin retrogradation (*13*, *14*).

Little is known about the effects of sourdough on various types of gluten-free bread. In a mixture of maize starch and flours from brown rice, soya, and buckwheat, sourdough fermentation resulted in protein degradation, which could be visualized by laser scanning confocal microscopy (LSCM).

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Upon incorporation of 20% of this sourdough, limited improvement of bread quality was detected (*15*). However, it remains unclear whether and according to which physicochemical mechanism protein degradation and bread quality are related in a gluten-free system. It is furthermore not sure whether these results can be extended to other gluten-free systems. In the previously mentioned study on gluten-free sorghum bread, sourdough fermentation did not have any impact on quality except flavor (*10*). This is in contrast to our own preliminary experiments with sorghum bread, which indicated clearly improved crumb structure upon sourdough incorporation. A decisive point in these experiments was that the total amount of sorghum flour was subject to sourdough fermentation, while pure starch was added afterward. Like that, sourdough fermentation could impact the complete sorghum flour while leaving the added pure starch unchanged. To supply sugars, a bacterial α-amylase was added that has also been shown to have antistaling properties in gluten-free starch bread (*16*). After sourdough fermentation, we neutralized part of the acidity by the addition of calcium carbonate, because excessive acidity had been found to be undesirable by U.S. consumers in informal taste panels.

The present article describes the production of this improved sorghum bread and investigates the physicochemical background of its quality by a variety of tests including fundamental rheology, LSCM of the bread crumb, and size-exclusion high-performance liquid chromatography (SE-HPLC) of the proteins. The starting point was a previously described bread based on sorghum flour and maize starch (70/30) without HPMC (*12*). A sequential scheme of replacing maize starch by potato starch, adding HPMC, adjusting the pH with lactic acid to the same value achieved by sourdough fermentation, and finally sourdough fermentation was used to understand the individual contributions of these ingredients to overall bread quality and structure.

MATERIALS AND METHODS

Breadmaking and Raw Materials. The tested formulations (**Table 1**) were (i) as described by Schober et al. (*12*) (control, abbreviated Co in this article); (ii) maize (corn) starch replaced by potato starch (PS); (iii) with HPMC added (wHPMC); (iv) with HPMC, bacterial α-amylase, and lactic acid in an equivalent amount as produced during sourdough fermentation (chemically acidified, CA); and (v) with HPMC, bacterial α-amylase, and the total amount of sorghum flour fermented for 24 h by *Lactobacillus plantarum* (sourdough treatment, SD). The CA treatment was chosen to test the combined effects of acidification, neutralization by calcium carbonate, and the bacterial α-amylase as in the SD treatment but without the contribution of a prolonged (24 h) fermentation time.

Commercially available sorghum flour from a tan plant, tannin-free, white-grained hybrid (Twin Valley Mills, Ruskin, NE) was used for all baking tests. The crude protein content ($N \times 6.25$) was 8.5% db (AACC method 46-30; *17*). The sourdough starter was a freeze-dried culture (*L. plantarum*, L2-1, $\geq 1 \times 10^{11}$ cfu/g, Danisco, Niebüll, Germany). HPMC (Methocel K4M, Food Grade, E 464) was obtained from Dow Chemical Co. (Midland, MI); the bacterial α-amylase used was Novamyl Conc. BG (Novozymes North America, Franklinton, NC), and crystalline l-(+)-lactic acid, unmodified regular corn starch, calcium carbonate, and calcium hydroxide were from Sigma (St. Louis, MO). Unmodified potato starch was from Bob's Red Mill (Milwaukie, OR). Skim milk powder, table salt (NaCl), sugar (saccharose), and active dry yeast were purchased locally. The particle size of maize starch, potato starch, and sorghum flour was determined by laser diffraction particle size analysis (LS 13 320, Beckman Coulter, Fullerton, CA).

The sourdough was prepared with a 300 W Kitchen Aid mixer (Ultra Power, St. Joseph, MI) equipped with a flat beater. Tap water, sorghum flour, skim milk powder, bacterial α-amylase, and starter culture were mixed for 30 s at the lowest speed, the mixer bowl was scraped, and mixing was continued for 90 s at level 2 out of 10. The mixing bowl was covered with plastic film and allowed to ferment at 30 °C for 24 h.

Table 1. Formulations for the Five Treatments[a]

	%				
treatment[b]	Co	PS	wHPMC	CA	SD
water	105	105	105	105	105
sorghum flour[c]	70	70	70	70	70
skim milk powder	1	1	1	1	1
maize starch[c]	30				
potato starch[c]		30	30	30	30
salt	1.75	1.75	1.75	1.75	1.75
sugar	1	1	1	1	1
active dry yeast	2	2	2	2	2
HPMC			2	2	2
bacterial α-amylase (1:10)[d]				0.01	0.01
L-(+)-lactic acid				2.41	
starter culture[e]					0.04
calcium carbonate				2.4	2.4

[a] Quantities on a 100% flour–starch basis. Actual experiments were based on 500 g of water and 333 g of sorghum (14% moisture basis) for one batch prepared in one bread machine. [b] Co [as Schober et al. (*12*)]; CA, chemically acidified with lactic acid in an equivalent amount as produced during sourdough fermentation and HPMC and bacterial α-amylase added; SD (water, complete amount of sorghum flour, skim milk powder, bacterial α-amylase, and starter culture prefermented for 24 h at 30 °C), main dough prepared with the addition of HPMC. [c] 14% moisture basis. [d] The commercial concentrated enzyme powder Novamyl Conc. BG was diluted 1:10 with potato starch for easier dosage. [e] Freeze-dried culture of *L. plantarum* ($\geq 1 \times 10^{11}$ cfu/g).

For dough mixing, fermentation, and baking, the procedure of Schober et al. (*12*) was modified in a way that it could be carried out in bread machines (Breadman Ultimate, Salton, Columbia, MO). Ripe sourdough or water (prewarmed to 30 °C) was first added into the bread pan (pan dimensions: length × width × height = 19 cm × 13 cm × 14 cm; volume ≈ 3100 mL). The remaining dry ingredients were carefully mixed before adding them to sourdough or water. This premixing was especially important when using HPMC, which tends to form lumps when moistened. The settings of the bread machines were as follows: preheat (5 min), knead 1 (at intervals, 3 min), knead 2 (continuous, 7 min), rise 1 (1 min), punch (1 min), rise 2 (1 min), shape (1 min), rise 3 (30 min), and bake (60 min at 355 °F ≈ 180 °C). The initial mixing and short rise/punch intervals were used to manually scrape down the bread pans and support homogeneous mixing, especially in formulations with HPMC. The temperature measured inside the bread machines during fermentation (rise 3) was ≈32 °C.

After baking, the breads were allowed to cool for 2 h prior to further analyses and packing. After determining the weights and volumes of the loaves, four 2.5 cm slices were cut from the center of each loaf using a slice regulator. The height of the center slice was measured, and three of the four slices were sealed individually into aluminized polyester resin bags (Mylar, 10.0" × 14.0", Impak, Los Angeles, CA) together with an oxygen absorber pad (2000 cm^3 capacity, Impak) to inhibit mold growth. Additionally, each bag was sprayed with ≈2 mL of 95% v/v ethanol directly before packing.

On days 0, 1, 4, and 7 (3, 24, 96, and 168 h after baking), the bread slices were assessed for crumb grain and texture, using a C-Cell (Calibre Control International Ltd., Appleton, Warrington, United Kingdom) and a texture analyzer (TA.XT.plus, Stable Micro Systems Ltd., Godalming, Surrey, United Kingdom), respectively. With the latter, texture profile analysis (TPA) of the crumb was conducted with a constant speed of 2.0 mm/s (pretest speed, test speed, and post-test speed) over a distance of 10.0 mm, corresponding to 40% compression of the 25 mm slices. The wait time between the first and the second compression cycle was 5 s, and the trigger force was 20.0 g. A 38 mm Perspex cylinder probe with blunted edges was used in conjunction with the 30 kg load cell. TPA hardness (peak force during the first compression cycle) and cohesiveness (ratio of the positive force area during the second

compression to that during the first compression) followed the definitions of Bourne (*18*).

LSCM of the Breadcrumb. Pieces (<1 mm^3) of bread crumb from wHPMC, CA, and SD breads were cut out of the loaf center 18 h after baking and placed on microscope slides. Following the procedure of Lee et al. (*19*), a weakly alkaline solution of fluorescein 5(6)-isothiocyanate (FITC) was added to the sample and allowed to dry at room temperature in a dark environment. After >1 h, immersion oil was dropped on top of the stained sample before adding the cover slip.

A Zeiss LSM 5 PASCAL (laser scanning confocal microscope) was used to image the bread crumbs. The LSM 5 system was equipped with a Zeiss Axioplan 2 MOT Research Microscope, a fully motorized stage, Plan Neofluor objectives (1.25×/0.035, 10×/0.3, 20×/0.5, 40×/0.75, and 40×/1.3 oil), a Plan Apochromat objective (63×/1.4 oil), and differential contrast interference. Fluorescence emission imaging of FITC was accomplished using the 488 nm line of a 458/488/514 argon gas ion laser to excite FITC. A secondary HFT 545 dichroic was used to split the emission signals into two signals. The shorter wavelengths passed through a band-pass 505−530 nm filter to image FITC fluorescence. For *z*-series, the Airy unit of the emission wavelength was adjusted to one giving an optical slice thickness of 0.7−0.9 μm, and this thickness was used as the slice interval. Between 15 and 21 layers of the fluorescence images were projected into one image representing a total thickness of 15 ± 1.5 μm.

Sourdough Analyses. The sourdough pH was measured with a pH probe directly in the sourdough because of its low viscosity and high dilution. In contrast, the pH of the final batters (complete formulations, **Table 1**) was measured in suspension (10.0 g of batter plus 100 mL of water). The total titratable acidity (TTA) of unfermented and ripe (24 h) sourdough was determined by suspending 10.0 g of sourdough in 100 mL of water and titrating with 0.1 M NaOH to pH 8.5 (with retitrating to pH 8.5, 5 min after it was first reached).

The sourdough consistency was determined by measuring the force required for extrusion of the sourdough through a defined nozzle as described previously (*12*) with the following modifications: The nozzle diameter was 3 mm instead of 10 mm, and the trigger force was reduced from 50 to 5 g. Both modifications were required because of the low viscosity of the sorghum sourdough. Three separate sourdough batches were prepared and measured after 2 h (to allow relaxation and temperature equilibration to 30 °C) and after 24 h. The 2 and 24 h measurements were repeated three times with each sourdough batch and averaged into one value per batch.

To study the protein changes in sourdough, a sequential extraction scheme, combined with SE-HPLC, was used. Three batches of sourdough (replicates) were prepared. Freshly prepared sourdough and ripe sourdough after 24 h of fermentation at 30 °C (SD0 and SD24, respectively) were examined. For this purpose, 2.0 g of SD0 and SD24 was centrifuged (15700*g*, 10 min; these centrifugation conditions were kept for all subsequent steps). The supernatant was filtered through a 0.45 μm polypropylene filter with glass fiber prefilter (PN 4559T, Pall Gelman Laboratory, Ann Arbor, MI), mixed 1 to 3 (v/v) with mobile phase [50 mM Na-phosphate buffer, pH 7.0, with 1% sodium dodecyl sulfate (SDS)], and again centrifuged. The supernatant (dough liquid, DL) was subject to SE-HPLC as described below.

A 200 mg amount of the pellet was transferred into a new vial and washed with 1 mL of distilled water (30 s of mixing and then centrifugation). Afterward, the pellet was extracted with 1 mL of SDS buffer, pH 10 (12.5 mM Na-borate buffer, pH 10.0, containing 2% SDS), for 30 min with continuous shaking. After centrifugation, the supernatant (SDS soluble proteins, SDS-Pr) was diluted 1 to 3 with mobile phase, centrifuged, filtered through 0.45 μm polyvinylidene fluoride filters (PN 4452, Pall Gelman Laboratory), and analyzed by SE-HPLC.

Next, the pellet was extracted by sonication (30 s at 10 W; Sonic Dismembrator F60, FM 1795, Fisher Scientific, Pittsburgh, PA), with 1 mL of the same SDS buffer, pH 10. Following centrifugation, the supernatant (sonicated protein, Son-Pr) was diluted with mobile phase, centrifuged, filtered, and analyzed by SE-HPLC as before.

For the next step, 2% of 2-mercaptoethanol was added to the SDS buffer, pH 10. The pellet was extracted with 1 mL of this reducing buffer for 30 min with continuous shaking. After centrifugation, the supernatant (reduced SDS soluble proteins, Red-Pr) was diluted with mobile phase, centrifuged, filtered, and analyzed by SE-HPLC as before.

It should be pointed out that DL is more concentrated than SDS-Pr, Son-Pr, and Red-Pr. DL results from sourdough when the water extracts the sorghum and skim milk powder. In sourdough, the ratio of sorghum plus skim milk powder to water is 68%. For SDS-Pr, Son-Pr, and Red-Pr, the calculated ratio of sorghum to aqueous solvent is 11%. Consequently, DL results from an about six times higher concentration of extractable solid matter.

SE-HPLC was conducted using an Agilent 1100 HPLC system (Quantum Analytics, Foster City, CA) with a BioSep-SEC-S3000 column (300 mm × 7.80 mm, Phenomenex, Torrance, CA). The mobile phase was 50 mM Na-phosphate buffer, pH 7.0, with 1% SDS. The column temperature was maintained at 40 °C, the flow rate was 1.0 mL/min, the injection volume was 50 μL, and detection was done at 214 nm.

For the determination of starch damage, sorghum flour and fresh and ripe sourdough (SD0 and SD24, respectively) were freeze-dried and then analyzed, following a modified AACC Method 76-31 (*17*). A commercial kit (Megazyme, Bray, Ireland) was used. Modifications included the use of 8 mL instead of 5 mL of dilute sulfuric acid to terminate the reaction in accordance with the instructions provided with the kit. Furthermore, centrifugation after addition of the sulfuric acid was extended by 10 min at 4000 rpm, because the sourdough samples were still cloudy after 5 min at 3000 rpm.

Fundamental Rheology. Batters PS, wHPMC, and SD were prepared according to the recipe in **Table 1**, omitting the yeast. Each lot was based on 50 g of water, dry ingredients were premixed, and the final batter was stirred briefly with a handheld kitchen mixer until homogeneous. For SD, the sourdough was fermented (at 30 °C for 24 h) and then, the remaining premixed dry ingredients were added as in case of the bread, with the exception that calcium hydroxide instead of calcium carbonate was used to raise the pH. This modification was required to avoid the formation of gas bubbles, which would affect rheological properties. The quantity of calcium hydroxide was selected so that the final pH values of the batters were the same as in case of the breadmaking procedure. All batters were allowed to rest for 2 h at room temperature before loading them into the rheometer. Experiments were done in duplicate, with a fresh batter for each replicate.

For rheological testing of the batters, a ViscoAnalyser 50 (Reologica Instruments, Lund, Sweden) equipped with an extended temperature cell (ETC-3) and serrated plate measuring system (25 mm diameter) was used. For gap control, the autotension function of the instrument was used with a target tension of 0.01 N. This function compensates for expansion or contraction of the sample under the influence of heating or cooling by enlarging or reducing the gap while keeping the normal force constant. The sample was loaded on the bottom plate, and the top plate was lowered to a gap between 3.1 and 3.2 mm, so that all of its area touched the sample. Excessive sample was trimmed off with a plastic knife, and the exposed edges were covered with high vacuum grease dissolved in hexane (about 1:5) with a small quantity of hydrophobic dye (Oil Red O) added. The dilution of the vacuum grease with hexane reduced viscosity and allowed for an easy application of this hydrophobic coating onto the edges of the soft batters. After evaporation of the hexane, a thin hydrophobic barrier resulted, reducing moisture loss and preventing drying and hardening of the exposed surfaces. The dye enabled us to verify both the uniform coverage of the edges and the absence of hydrophobic coating between sample and plates. In clear contrast to mineral oil, there was no tendency that the grease might get between the sample and the plates and cause wall slippage.

After sample loading, 10 min of relaxation in the rheometer was allowed before the start of the measurement. Then, dynamic oscillatory testing at 1 Hz in the linear viscoelastic region was conducted with a linear temperature gradient (25−95 °C in 47 min), followed by 10 min at 95 °C, followed by a linear gradient down from 95 to 25 °C in 47 min. The target strain was 5×10^{-4}, which was in the linear viscoelastic region, based on stress sweeps with batters PS and wHPMC at 30 and 70 °C.

Additional rheological experiments were conducted with the same procedure (except for HPMC solution, see directly below), but using

Table 2. Baking Results[a]

	Co	PS	wHPMC	CA	SD	LSD[b]
appearance	always collapsed	inconsistent (once acceptable, twice collapsed)	tendency to have hole in center of crumb, flat to collapsed top	acceptable, but small volume, slightly collapsed top	overall good, round top	
specific volume (cm^3/g)			2.60 a	2.25 b	2.68 a	0.13
height (cm)			10.7 b	9.3 c	12.2 a	1.1
batter pH		6.10 a	6.12 a	5.18 b	5.18 b	0.04

[a] Within each row, numbers not sharing a common lowercase letter are significantly different ($P < 0.05$). [b] LSD for $P < 0.05$.

modified batters, to study the effect of individual ingredients on rheological properties. The modifications in composition of these batters are detailed together with the results. The rheology of pure HPMC (HPMC solution) was studied by first suspending it in boiling water. Upon cooling, a viscous solution formed. Evaporated water was then replaced to reach the same HPMC/water ratio as in the batters, and salt and sugar were added to create comparable concentrations of ions and soluble low molecular weight substances as in the batters. Because this solution was thinner than the batters, serrated plates were replaced by smooth 25 mm plates, and the gap was reduced to 1 mm. Dynamic oscillatory testing was done with the same temperature gradient as described for the batters but at an increased strain of 5×10^{-3}. This latter strain was in the linear viscoelastic region of the HPMC solution, as determined by stress sweeps.

Statistical Design and Analyses. Breadmaking experiments followed a randomized block design with three blocks. Each block corresponded to one replicate, within which the treatments were conducted in random order. Experiments for each block were finished before the next block was started. Random blocks were incorporated into the general linear model, and least significant differences (LSDs) were based on individual error rates. Specific volume, bread height, batter pH, and the TPA parameters were analyzed in this manner.

Other experiments, including particle sizes of starches and sorghum flour, fundamental rheology with PS, wHPMC, and SD, starch damage, and sourdough consistency by extrusion were done in one block, following completely randomized designs. For analysis, individual error rates were used, and equal variances for the compared samples were assumed.

RESULTS AND DISCUSSION

Sourdough Fermentation and Breadmaking. Preliminary results suggested that sorghum sourdough started with *L. plantarum* L2-1 did not acidify reliably unless a small portion (≈1%) of skim milk powder was added. We therefore incorporated it into all treatments (Co, PS, wHPMC, CA, and SD; **Table 1**) to maintain comparable conditions. Sourdough prepared with skim milk powder (**Table 1**) reached pH 6, 5, and 4 in about 4, 7, and 11 h, respectively. After that, the pH dropped only slowly to 3.7 ± 0.03 at 24 h (average ± standard deviation). TTA of the ripe (24 h) sourdough averaged 17.1 ± 0.1 mL of 0.1 M NaOH, while unfermented sourdough had a TTA of 2.1 ± 0.3 mL. The difference (15 mL) originates from acids formed during fermentation. *L. plantarum* is a facultatively heterofermentative lactic acid bacterium. From pentoses, considerable amounts of acetic acid are formed. In contrast, hexoses are fermented homofermentatively, and almost only lactic acid is formed (*20*). In sorghum, the total amount of pentosans and thus potential free pentoses is low (*12, 21*); therefore, lactic acid is the predominant acid. The TTA value of 15 mL was used to calculate the amount of lactic acid required for chemical acidification of the CA treatment to the same pH as upon incorporation of sourdough. It was also the basis for the calculation of the amount of calcium carbonate added (neutralization of 90% of the lactic acid on a molar basis in CA and SD treatments). The pH values of the final batters (after addition of all ingredients, **Table 1**, including calcium carbonate in the case of CA and SD) confirmed the correctness of these calculations (**Table 2**). SD and CA batters had identical average pH values, which were significantly lower than those of wHPMC and PS batters due to neutralization of only 90% of the acid.

Major baking results are summarized in **Table 2**. The Co bread was collapsed in all cases. This is in contrast to a previous study (*12*), in which this formulation yielded acceptable bread. An important difference between the previous and the present study was the larger size of the bread pans in the present study (3100 vs 875 mL). Upon proofing and baking before and during starch gelatinization, the batter is very soft. While mechanical support comes from the side walls of the pan, the batter toward the center of the pan is only supported by surrounding batter. In the case of a larger pan of similar shape than a smaller one, the increase in batter volume and mass is proportional to the third power of the length, while the increase of the area of the supporting side walls is only proportional to the length squared. This means that in larger pans relatively more weight has to be supported by the batter itself. This fact is likely to facilitate collapsing, especially of the crumb center in larger batter-based breads.

While preliminary tests indicated better bread quality when potato starch was used instead of maize starch, in the main tests, there was a tendency of the breads to collapse also when potato starch was used, and results were inconsistent between replicates (PS; **Table 2**). A slightly better performance of potato starch might be explained by its lower gelatinization temperature in comparison to maize starch (*22*). Earlier gelatinization would result in an earlier increase of the batter/crumb consistency during baking. In contrast, a relatively low consistency of the batter/crumb upon baking over prolonged time periods would facilitate collapsing. Rheology indeed showed a much earlier gelatinization of the potato relative to the maize starch used in this study (see below under Fundamental and Empirical Rheology). The inconsistent results specifically for the PS bread suggest that this bread was in all cases at the edge of collapsing, so that small random effects determined the result. These might include fluctuations in room temperature and air pressure, yeast activity, or the timing of the heating cycles of the bread machine. Because of the slightly better results despite inconsistencies, we decided to use potato starch instead of maize starch for subsequent experiments.

The addition of HPMC (wHPMC; **Table 2**) resulted in significant improvements of bread quality and in more consistent results. This improving effect of HPMC on sorghum bread was already described by Hart et al. (*10*). In contrast to the present study, these authors did not find the problem of a hole in the crumb center. However, their experiments were based on pup loaves and 180 g of batter, in contrast to the regular pan size

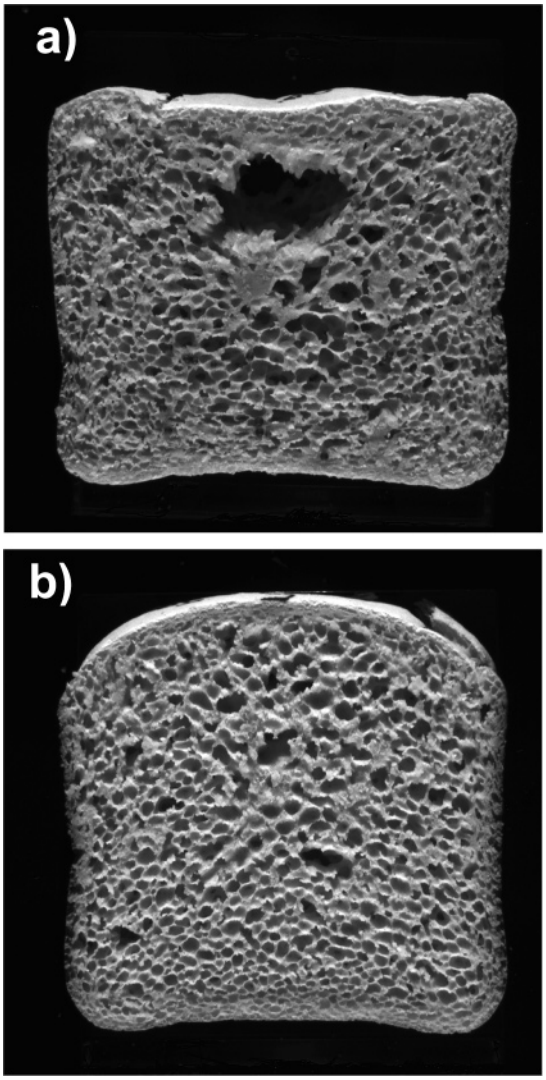

Figure 1. Bread slices from (**a**) wHPMC and (**b**) SD treatments (**Table 1**). Both were from the same replicate. Notice the hole with the collapsed crumb area directly below in the wHPMC treatment and the round top resulting in increased height in the SD treatment.

and >1000 g of batter used in the present study (**Table 1**, footnote a). This increased size is again a greater challenge for the stability of the crumb structure upon proofing and baking but is essential for the successful production of regular-sized gluten-free bread in an industrial process.

Chemical acidification of HPMC-containing bread (CA; **Table 2**) resulted in a small loaf volume relative to nonacidified bread (wHPMC). In clear contrast, bread in which all of the sorghum flour had been subject to sourdough fermentation (SD) showed a good volume (practically identical to that of wHPMC bread). However, in the case of the SD bread, there was no hole in the crumb center found and the height was significantly improved relative to the wHPMC bread. This increased height in combination with a similar volume to the wHPMC bread is a result of the round top of the SD bread vs the flat top of the wHPMC bread. **Figure 1** shows the major differences between the wHPMC and the SD bread. To minimize the chance that the quality of the SD bread was only superior due to random effects, we rebaked the SD treatment five more times and evaluated the results qualitatively. The described superior quality was confirmed.

It is noteworthy that the pH of the batter was identical between the CA and the SD breads (**Table 2**). Because of the

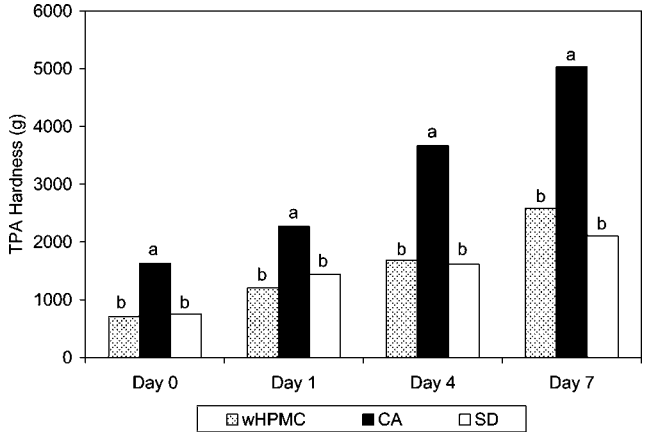

Figure 2. Crumb firmness (hardness value from TPA) for the three treatments wHPMC, CA, and SD (**Table 1**) over 7 days of storage. Different lower case letters indicate significant differences ($P < 0.05$) within each storage time. LSDs increased over storage time (308, 610, 1053, and 1895 g for days 0, 1, 4, and 7, respectively).

inferior quality of the CA bread, pH effects alone can be excluded as a factor for the improved quality of the SD bread relative to wHPMC. TPA (**Figure 2**) showed a slightly lower staling rate for the SD relative to the wHPMC bread within 7 days of storage; however, the differences between the crumb firmness (TPA hardness) of these two breads were not significant at any given storage time. Among other differences, the SD bread unlike the wHPMC bread contained the bacterial α-amylase with supposed antistaling effects (*16*). We are currently examining the limited efficiency of this enzyme in the SD formulation. Possible explanations include the inactivation of the enzyme during the long incubation time at low pH in the sourdough and an insufficient dosage for efficient prevention of staling. Preliminary experiments showed a high sensitivity of the sorghum bread to overdosage of the amylase. It appears possible that in pure starch breads as described in ref *16*, a higher dosage is possible. According to our observations, starch breads are overall more stable than batter-based breads from flours of gluten-free cereals. The considerable hardness of the CA bread (**Figure 2**) reflects its low volume. As in the case of the SD bread, no positive effect of the addition of the bacterial α-amylase could be noted.

Crumb cohesiveness (data not shown) decreased during storage for wHPMC, CA, and SD breads, indicating that the breads became more brittle. At day 7 of storage, the cohesiveness was significantly ($P < 0.05$) better for the SD bread than for both other types (0.39 vs 0.34 and 0.31 for SD, wHPMC, and CA, respectively). A further potential advantage of the SD bread was that its crumb subjectively appeared less gritty in the mouth, which might indicate some degradation of coarse particles during sourdough fermentation. More research is required to verify this specific point.

Fundamental and Empirical Rheology. To understand the microstructure that contributes to bread quality, fundamental rheology was conducted with a temperature gradient (25−95 °C in 47 min, 95 °C for 10 min, and 95−25 °C for 47 min), simulating the baking and cooling process. However, despite hydrophobic coating of the sample, its edges dried considerably during the 95 °C holding period and subsequent downward gradient. Therefore, only the first 50 min were plotted, during which sample drying was not a major problem. Data in **Figure 3** allow the identification of the contribution of individual recipe components, while **Figure 4** compares the three treatments PS, wHPMC, and SD. **Table 3** lists the particle sizes of the flour

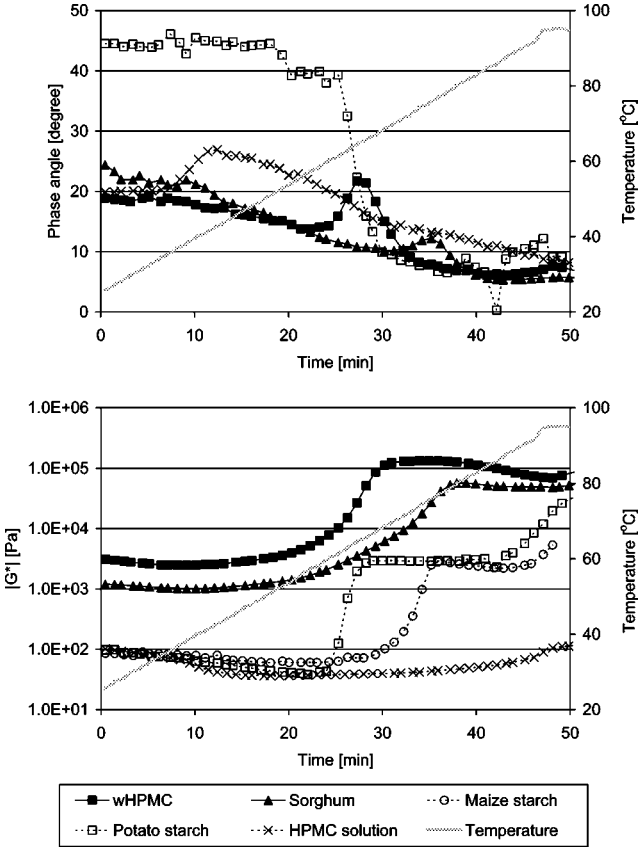

Figure 3. Contribution of the individual ingredients to the fundamental rheological properties of batter wHPMC (**Table 1**). Dynamic oscillatory temperature sweeps at 1 Hz within the linear viscoelastic region are plotted, where phase angle characterizes the degree of elasticity (lower = more elastic) and $|G^*|$ characterizes the resistance to deformation (higher = firmer). The tested samples were as follows: HPMC solution (water, salt, sugar, and HPMC), potato starch and maize starch (HPMC solution plus the respective starch), sorghum (HPMC solution plus sorghum flour), and wHPMC. All ingredients for all samples were in ratios as in **Table 1**; the phase angle for maize starch was omitted to improve readability.

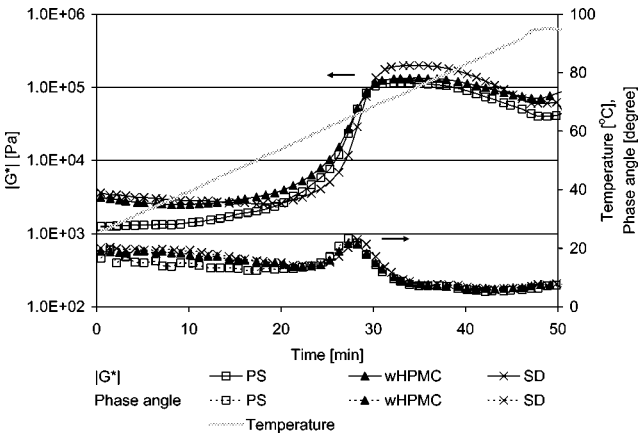

Figure 4. Dynamic oscillatory temperature sweeps at 1 Hz within the linear viscoelastic region for batters PS, wHPMC, and SD (**Table 1**). For $|G^*|$ and phase angle, see **Figure 3**. $|G^*|$ is significantly ($P < 0.05$) lower for PS than wHPMC and SD at 31 ± 0.4 °C and significantly ($P < 0.05$) higher for SD than PS and wHPMC at the respective peak value after gelatinization (72.1–76.2 °C).

and starches to better understand their contribution to rheological behavior on a micro- to millimeter range. As expected, potato starch granules were larger than maize starch granules, and the median values (50% volume) agree well with the literature (*22*). The distinctly larger particle size and broader size distribution of the sorghum flour indicate that a considerable portion of the starch is still embedded in the protein matrix. This is in agreement with the observations of Duodu et al. (*23*) on hammer-milled sorghum flour, who found large groups of unbroken cells from vitreous and floury endosperm as well as subcellular fragments composed of starch and protein bodies. Individual sorghum starch granules have a diameter of about 35 μm (*22*).

Table 3. Particle Size of Starches and Sorghum Flour[a]

	particle diameter (<μm)		
volume (%)	maize starch	potato starch	sorghum flour
10	8.1 a	20.5 b	21.7 c
50	13.9 a	41.1 b	118.6 c
90	21.3 a	67.2 b	276.6 c
99	30.8 a	94.1 b	395.1 c

[a] Determined by laser diffraction particle size analysis. Within each row, all results were significantly different at $P < 0.001$, except for potato starch vs sorghum flour at 10% ($P < 0.05$).

Assuming that the rheological behavior of batter wHPMC reflects largely the properties of its major components, a stepwise investigation from simple to more complex systems will be discussed in the following order: HPMC solution, starch suspensions, sorghum flour suspension, and finally batter wHPMC (**Figure 3**). Prior to this analysis, we verified and confirmed that the small amount of skim milk powder (**Table 1**) did not affect rheological behavior of PS batter and a pure sorghum batter (wHPMC batter without potato starch) (data not shown). The HPMC solution (**Figure 3**) contained water, salt, sugar, and HPMC in the same ratio as in wHPMC (**Table 1**) and was the only sample prepared by mixing the HPMC into boiling water and then letting it cool, due to the absence of any other powder to dilute the HPMC. This solution was highly viscous at room temperature. **Figure 3** shows a comparatively high $|G^*|$ (absolute value of the complex dynamic shear modulus) value (≈100 Pa) and low phase angle (20°) at 25 °C, indicating notable resistance to deformation and a considerable degree of elasticity, respectively. (Purely elastic behavior would correspond to a phase angle of 0, and purely viscous behavior would correspond to a phase angle of 90°). Increasing the temperature at first weakened the viscous HPMC solution, as indicated by a decrease of $|G^*|$ and an increase in phase angle. However, beyond about 30 min, corresponding to roughly 70 °C, $|G^*|$ increased again and the phase angle had reached values below 15°, which thereafter only dropped slowly. Heating the same HPMC solution in a beaker showed the appearance of turbidity at about 60 °C and aggregation and formation of solid gel strands at and above 65−70 °C. This temperature corresponds to the hot gel formation temperature provided by the supplier (70−90 °C). For a further understanding of the effects of HPMC, we examined its surface-active properties. When a solution of 1.9% HPMC in water (corresponding to 2 parts per 105 parts of water; **Table 1**) was mixed in a blender, so that lumps were dispersed and air could be incorporated, a stable foam was formed, resembling whipped egg white. At a reduced concentration of 0.5%, the resulting dispersion resembled a solution of laundry detergent, with a foam layer on top and a clear liquid at the bottom. These observations clearly show that the HPMC type used in this study was distinctly surface-active. If the HPMC concentration is sufficiently high, the increased viscosity of the liquid phase causes the air bubbles

to be dispersed throughout the liquid; if it is lower, the foam rises to the top. The surface activity of methylcellulose and HPMC has been described in the literature and is a characteristic difference to xanthan gum, which is not surface-active (*24*). Gas cell stabilization by surface-active substances has been recognized as an important factor in wheat bread despite the presence of gluten (*25*). It becomes even more central in batter-based gluten-free bread, similar to surface-active egg protein in cake, preventing coalescence of bubbles (*8*).

Maize and potato starch batters were composed as the HPMC solution plus the respective starch in the amount listed in **Table 1**. Upon gelatinization, both starches caused a strong increase in $|G^*|$ and a strong decrease in phase angle (**Figure 3**, phase angle for maize starch omitted to improve readability). Comparable rheological behavior of wheat starch in wheat dough and starch−gluten−water blends has been described previously (*26*, *27*). In agreement with Lineback (*22*), maize starch gelatinized at a higher temperature than potato starch. Sorghum batter was prepared as the starch batters, except that starch was replaced with sorghum flour in the amount listed in **Table 1**. The higher solid matter content of the sorghum batter relative to the starch batters resulted in higher $|G^*|$ values over the whole temperature range shown and lower phase angles until about 30 min. The batter also differed from the starch batters in that the increase in $|G^*|$ and decrease in phase angle was over a much broader range, reflecting the broader particle size distribution of the sorghum flour and the presence of particles in which starch was still embedded in endosperm cells or at least a protein matrix, as described above. Under such conditions, the swelling and gelatinization of the starch granules may be restricted (*28*), and embedded starch can be assumed to gelatinize at higher temperature roughly as the particle size increases. Noteworthy is a peaklike increase and subsequent decrease of the phase angle toward the end of gelatinization. A similar, but larger, peak at lower temperatures can be seen in batter wHPMC. This batter can be regarded as the sorghum batter just described plus potato starch and skim milk powder (**Table 1**). Consequently, the increased dry matter content relative to the pure sorghum batter is reflected in higher $|G^*|$ values, lower initial phase angles, and a steepness of $|G^*|$ upon gelatinization between sorghum batter (flat) and potato starch batter (steep). The marked peak in phase angle just before 30 min agrees well with the gelatinization range of potato starch. Further evidence that it was caused by the gelatinization of the potato starch was obtained by an additional experiment, where the batter wHPMC had the potato starch reduced to 1/3 of the normal amount. The peak was still there but smaller than in the regular wHPMC batter. A possible explanation for both the smaller peak of the phase angle in sorghum batter and the larger peak in wHPMC batter is that initially the sorghum flour particles stick loosely together. These aggregated particles would be stable enough to achieve considerable elasticity at the small alternating deformations of the dynamic oscillatory measurements, resulting in phase angles of about 20°. Upon starch gelatinization, the aggregated particles would be separated by the expanding starch granules, and elasticity would decrease (i.e., phase angle increase), until a sufficiently stable starch gel would be formed to compensate for this loss of elasticity. This might indeed be a very critical stage of the baking process, where collapse might occur, although further research has to verify these hypotheses.

During fermentation, sourdough became thinner. This observation was confirmed by consistency measurements, in which the sourdough was extruded through a nozzle and the force was recorded. The extrusion force between a fresh (2 h) and ripe

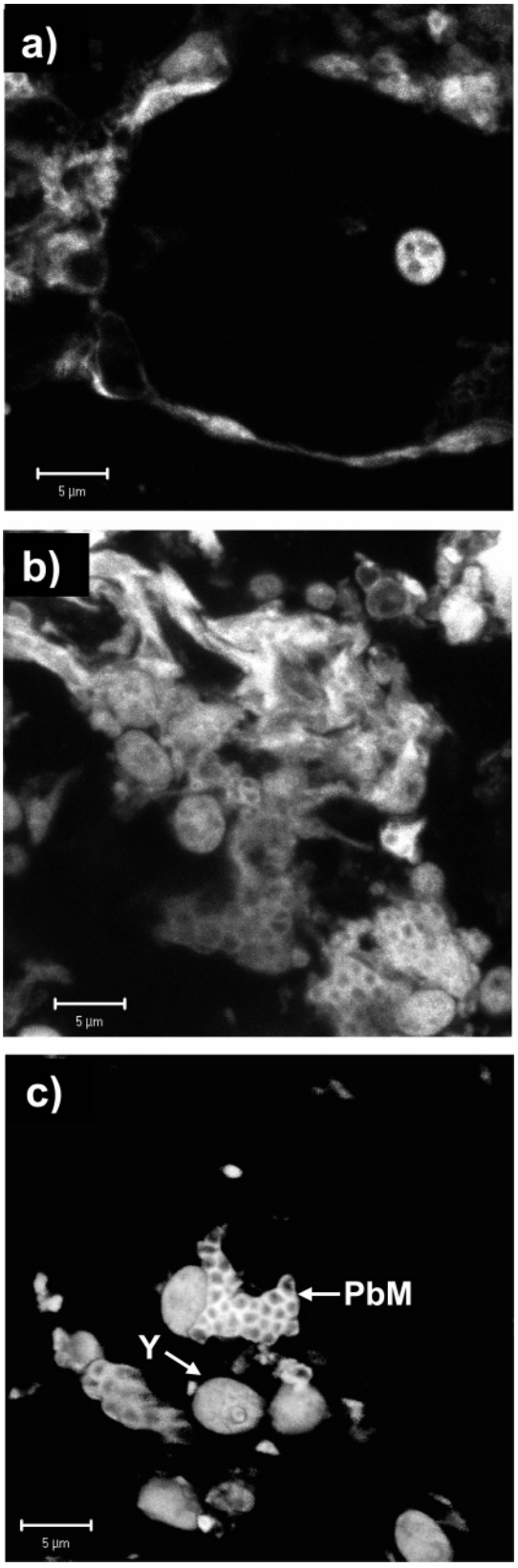

Figure 5. LSCM images of bread crumbs from (**a**) wHPMC, (**b**) CA, and (**c**) SD (**Table 1**). Proteins were selectively stained with the fluorescence dye FITC and appear bright in the images. Yeast cells (Y) and protein bodies still embedded in their matrix (PbM) are marked. Note that aggregated protein is virtually absent in SD crumb, while it is present in medium and large amounts in wHPMC and CA crumbs, respectively. Magnification bars correspond to 5 μm, and images represent an approximate depth of 15 μm due to projection of vertical layers into one image.

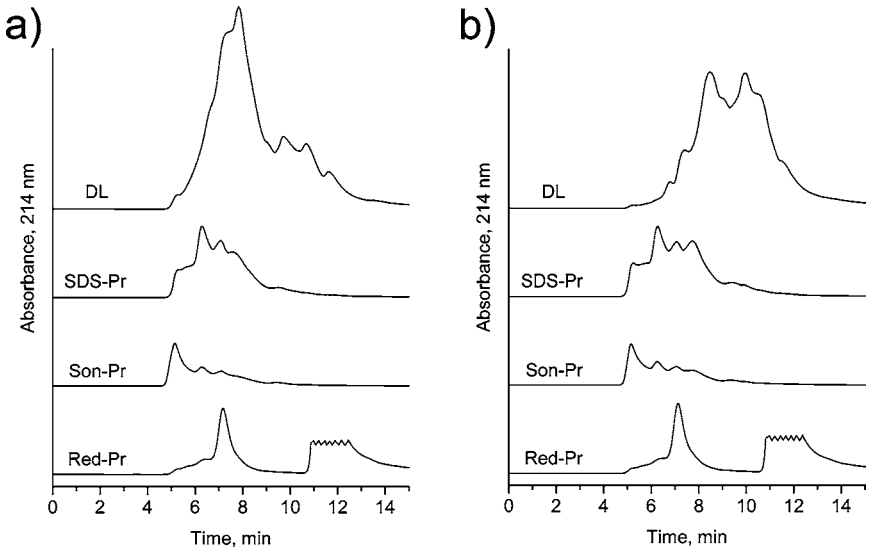

Figure 6. SE-HPLC of protein fractions from (**a**) freshly prepared and (**b**) ripe (24 h fermented at 30 °C) sorghum sourdough (**Table 1**). DL, proteins from dough liquid after separation from nonsolubilized solids (pellet); SDS-Pr, Son-Pr, and Red-Pr, proteins sequentially extracted from pellet with SDS buffer, pH 10, SDS buffer, pH 10, with sonication, and reducing SDS buffer, pH 10, respectively. The solvent peak in Red-Pr fraction (>10.5 min) has been truncated. DL is by about a factor 6 more concentrated than the other fractions and appears therefore enlarged. SE-HPLC at 40 °C on a BioSep-SEC-S3000 column; mobile phase, 50 mM Na-phosphate buffer, pH 7.0, with 1% SDS; flow rate, 1.0 mL/min.

(24 h) sourdough differed by 48% and was 4.6 N for fresh vs 2.4 N for ripe sourdough ($P < 0.01$). Factors causing such a drop in consistency during fermentation might include the degradation of mechanically damaged starch by amylases from sorghum and the added bacterial α-amylase and degradation of proteins. Protein changes will be addressed below separately. Starch damage of sorghum flour, fresh sourdough (0 h), and ripe sourdough (24 h) was 13.7, 12.7, and 11.5% db, respectively, and all differences were significant ($P < 0.01$). Although these changes pointed in the expected direction, the differences were relatively small and could most likely not be the only cause of the drop in consistency during sourdough fermentation. (The difference between flour and fresh sourdough may be explained by some enzymatic degradation of damaged starch between sourdough preparation and complete freezing of the sample.)

Oscillatory tests at increasing temperature as described above were conducted with batters PS, wHPMC, and SD (**Figure 4**). The phase angle showed no notable differences over the whole time and temperature range plotted. $|G^*|$ below 50 °C was lowest for PS batter, as can be expected when viscosity-increasing HPMC is absent. Statistical tests at 31 ± 0.4 °C, equivalent to fermentation temperature, showed that $|G^*|$ was significantly ($P < 0.05$) lower for PS batter than for wHPMC and SD batters, while there was no significant difference between the latter two. In contrast, after gelatinization (>65−70 °C), $|G^*|$ was highest for SD batter. Statistical analysis showed that temperatures, at which the peak value of $|G^*|$ was reached, averaged between 72.1 and 76.2 °C for the three batters, and these temperature differences were not significant. Comparison of the peak values of $|G^*|$ showed a significantly ($P < 0.05$) higher value for SD batter than for PS and wHPMC batters, while the latter two were not significantly different. It has been found previously by a different rheological technique (Newport Rapid Visco Analyzer) that lactic acid fermentation of a sorghum flour increased peak viscosity upon heating of a suspension of this flour (*29*). Sourdough fermentation of sorghum flour therefore seems to cause a stronger starch gel upon subsequent heating.

Molecular and Microscopic Aspects. Sourdough fermentation caused a more stable crumb structure of the breads, in that it prevented the formation of a hole in the crumb and increased loaf height (**Figure 1**). Both effects might be a result of the stronger starch gel. For a further understanding of the mechanisms, we compared bread crumbs of wHPMC and SD breads on a microscopic level and also included CA bread crumb to address the effect of acidification alone. **Figure 5** shows fluorescence images obtained by LSCM, representative for each bread crumb. Proteins appear bright due to selective staining with a protein fluorescence dye (FITC). Preliminary experiments with a pure wheat starch bread showed that gelatinized starch was not stained and was not visible in the LSCM fluorescence images. Individual components are easiest identified in the SD bread crumb. Only yeast cells (Y) and protein bodies (less intense) embedded into an intensely fluorescenting matrix (PbM) are visible. The size of the individual protein bodies (≈1 μm) agrees well with other studies, applying electron microscopy (*23*, *30*−*32*). Results from one of these studies (*23*) suggest that in sorghum flour, protein bodies held together by matrix protein originate from the vitreous endosperm. Another study (*30*) reported that in ungerminated sorghum, protein bodies cannot significantly hydrolyze themselves. Only upon addition of proteinase extract from germinated sorghum was the matrix protein, and to a lesser degree the protein bodies themselves, degraded. In the present sourdough fermentation, no isolated protease or malted sorghum was added; therefore, the presence of protein bodies embedded in matrix protein can be expected. These might contribute to some gritty mouthfeel even after fermentation.

In the wHPMC bread crumb, yeast and protein bodies embedded in matrix protein were also visible, although somewhat masked by strands and lumps of aggregated protein. Large amounts of aggregated protein became the dominant structure element in the CA breadcrumb, although protein bodies in their matrix and yeast cells were also visible. The results shown in **Figure 5** suggest that unlike protein bodies, the specific sourdough fermentation of this study could degrade some other proteins, which would otherwise aggregate upon baking as in the case of wHPMC crumb. The formation of oligomers or polymers through disulfide bonding upon cooking of sorghum has been described in various studies (*23*, *31*, *33*). In the case

of the CA treatment, lowering the pH to about 5.2 without allowing extra incubation time greatly promoted protein aggregation. Poor solubility of sorghum proteins at acidic pH values has been described previously (*34*, *35*). In contrast to gluten proteins, aggregated proteins in case of wHPMC and CA crumbs were clearly not associated with improved bread quality. In contrast, it appears possible that the superior strength of SD batter upon heating originated from less interference of protein with the starch gel.

For an in-depth understanding of the protein changes upon sourdough fermentation and verification of the results of microscopy, samples of fresh (0 h) and ripe (24 h) sourdough were analyzed by a sequential extraction/SE-HPLC procedure (**Figure 6**). The analyzed fractions were the original dough liquid (DL) after separating from nonsolubilized solids (pellet), and proteins sequentially extractable from the pellet: proteins soluble in SDS buffer pH 10 (SDS-Pr), proteins soluble in SDS buffer after sonication (Son-Pr), and proteins soluble in reducing SDS buffer (Red-Pr). The three replicates were qualitatively identical, and all chromatograms in **Figure 6** are from the same replicate that quantitatively represents medium properties of the three replicates. It should be noted that DL is about a factor of 6 times more concentrated than the other fractions and therefore appears enlarged in **Figure 6**. Major changes due to the 24 h sourdough fermentation occurred only in the DL fraction, while changes in the other fractions (SDS-Pr, Son-Pr, and Red-Pr) were only minor. In the DL fraction, a shift toward smaller sizes in the ripe sourdough was obvious. For an estimation of molecular masses, it was assumed that monomeric, rather than polymeric, kafirins dominate in the reduced (Red-Pr) fraction, and these have been reported to have molecular masses between 19 and 27 kDa (*32*). Also, the major peak in the Red-Pr fraction, at 7−8 min, lies reasonably in this molecular mass range when compared to SE-HPLC data reported previously (*36*). Thus, after sourdough fermentation, the bulk of the DL fraction (elution time >8 min) is degraded to molecules smaller than kafirin monomers. These results suggest that proteases mainly degrade proteins that are already soluble at the beginning of fermentation. Degradation of those proteins soluble in the DL to smaller peptides may well explain why they can no longer cross-link and therefore do not aggregate upon baking. It may also be another reason, aside from some degradation of damaged starch, that sourdough consistency decreased upon fermentation.

The finding that mainly proteins soluble in the aqueous DL are degraded is in clear contrast to studies on rye sourdough, where especially the prolamins (secalins) soluble in 70% ethanol and less so the water-soluble proteins were degraded (*37*). This difference may be explained by the limited accessibility of the sorghum prolamins (kafirins) to proteases, due to their location primarily in stable protein bodies. Especially the main prolamin fraction, α-kafirin, is not easily degraded by proteases because of its location in the interior of the protein bodies (*31*).

In conclusion, sorghum bread of superior quality relative to previously described formulations was produced in the present study. Keys to its quality were the use of the surface-active hydrocolloid HPMC and sourdough fermentation of the total amount of sorghum. One major effect of this sourdough fermentation was the degradation of proteins soluble in the DL. Undegraded, these proteins would aggregate upon baking and interfere with the starch gel. As a consequence, bread without sourdough fermentation tended to have a large hole in the crumb center.

ABBREVIATIONS USED

|*G**|, absolute value of the complex dynamic shear modulus; CA, chemically acidified treatment (**Table 1**); Co, control treatment (**Table 1**); FITC, fluorescein 5(6)-isothiocyanate; HPMC, hydroxypropyl methylcellulose; LSCM, laser scanning confocal microscopy; LSD, least significant difference; PS, treatment where maize starch was replaced by potato starch (**Table 1**); SD, sourdough treatment (**Table 1**); SDS, sodium dodecyl sulfate; SE-HPLC, size-exclusion high-performance liquid chromatography; TPA, texture profile analysis; TTA, total titratable acidity; wHPMC, treatment with HPMC added (**Table 1**).

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