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Removal of surface lipids improves the functionality of commercial zein in viscoelastic zein-starch dough for gluten-free breadmaking☆

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ABSTRACT

Maize prolamin (zein), together with starch, hydroxypropyl methylcellulose, sugar, salt, yeast and water can form wheat-like cohesive, extensible, viscoelastic dough when mixed above room temperature (e.g. 40 °C). This dough is capable of holding gas. However, it is excessively extensible, and when used for hearth-type rolls, it tends to become flat. Bench-scale defatting of zein with chloroform at room temperature significantly ($P < 0.05$) improved specific volume (4.5 ml/g vs. 3.3 ml/g) and shape of the rolls (width-to-height 2.0 vs. 3.9). The total lipid content determined by accelerated solvent extraction (100 °C, 69 bar, chloroform), however, only decreased from 8.0 to 6.6% due to this bench-scale defatting. Staining experiments with Naphthol Blue Black suggested that bench-scale defatting removed surface lipids from the zein particles, and thus facilitated their aggregation. Aggregation experiments with zein and water at 40 °C, and laser scanning confocal microscopy with zein-starch dough confirmed that zein particles aggregated more easily when surface-defatted. Dynamic oscillatory temperature sweeps demonstrated that surface-defatting lowered the temperature at which protein cross-linking occurred by 2 °C. This research can help to produce superior gluten-free bread and could also possibly contribute to the better understanding of wheat dough.

1. Introduction

Celiac disease, an immune-mediated enteropathy, requires a strict gluten-free diet, and thus forbids, among many other food products, the consumption of wheat and rye bread. Together, increased awareness for the disease and changing eating habits are causing a rapid growth of the market for gluten-free products worldwide. Based on screening data, the prevalence of the disease may be close to 1% of the population of many countries around the world, emphasizing the importance of this market (Cureton and Fasano, 2009).

Gluten-free bread production has long been studied, and the decisive discoveries such as the use of suitable hydrocolloids, and optimization of formulations in terms of volume and crumb grain were achieved several decades ago (e.g. Nishita et al., 1976; Ranhotra et al., 1975). These breads are made from soft doughs resembling batters, and, as such, their manufacture is different from regular wheat bread. For example, rather than molding the gluten-free doughs, they are usually poured in bread pans. Hearth-type breads and rolls can typically not be produced from them. Because of the absence of an aggregated gluten network, the batter-like dough consistency and the rigid texture of the finished loaves, these breads have been compared to cakes (Cauvain, 1998; Taylor and Dewar, 2001) or German-style rye breads (Schober, 2009).

Recently, a new system based on maize prolamins (zein) has been successfully used for gluten-free bread production (Schober et al., 2008). This idea goes back to the discovery that zein-starch mixtures form viscoelastic doughs closely resembling wheat dough, provided that they are mixed above room temperature (Lawton, 1992). In addition to this physical similarity between zein-starch dough and wheat dough – best characterized as cohesive, extensible and viscoelastic – there are also structural similarities. Both, Lawton (1992) and Schober et al. (2008) described gluten-like zein strands forming a network in the zein-starch dough. Besides the

Abbreviations: ASE, accelerated solvent extraction; ELSD, evaporative light scattering detector; FITC, fluorescein 5(6)-isothiocyanate; G′, storage modulus; G″, loss modulus; HPMC, hydroxypropyl methylcellulose; LSCM, laser scanning confocal microscopy; SDS, sodium dodecyl sulfate; SE-HPLC, size-exclusion high-performance liquid chromatography; T_g, glass transition temperature; Z1, zein lot No. 1; Z1, Hexane/Z1, $CHCl_3$; Z1, bench-scale defatted with hexane and chloroform, respectively; Z2, zein lot No. 2.

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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need for higher temperature, zein-starch dough tends to differ from typical wheat dough mainly by being weaker and more extensible (Lawton, 1992; Schober et al., 2008). Consequently, in our preliminary experiments, baked products produced without pans tended to become flat upon proofing and baking, even though the dough could be well-shaped. In our previous study (Schober et al., 2008), therefore, zein-starch dough still had to be baked in bread pans. However, continuing work showed distinct differences between two lots of zein: a new zein lot resulted in zein-starch dough with much superior strength, which tended to produce improved, less flat hearth-type rolls. The objective of the present study is to identify the physicochemical factors responsible for the superior performance of the new zein lot. As a starting point, the similarities between zein and wheat gluten suggest that a comparison of both systems might be helpful.

In wheat, it is widely accepted that the molecular weight distribution of the gluten proteins controls dough properties (Southan and MacRitchie, 1999). As summarized by these authors, the molecular weight distribution is determined by the ratio of monomeric proteins (largely gliadins) to polymeric proteins (largely glutenins), and the size distribution of the polymeric proteins. Large, predominantly linear polymers are made up from glutenin subunits, which are covalently (disulfide) linked with each other. These linear polymers form molecular entanglements resulting in elasticity. Size distribution of the polymeric proteins, in turn, depends on the properties of the high molecular weight glutenin subunits (HMW-GS) from which they are made. The relatively recent hyper-aggregation model (Hamer and Van Vliet, 2000) expands this view beyond the molecular level. The polymers from covalently linked glutenin subunits (molecular level), are supposed to further aggregate via mesoscopic particles into macroscopic aggregates, involving non-covalent interactions and disulfide bridges.

Unlike the importance of proteins for the quality of wheat dough, the role of lipids is less clear cut. For example, it has been suggested that lipoproteins might contribute to the softness and plasticity of gluten by forming slip planes within the gluten (Grosskreutz, 1961). Others, in contrast, suggested that lipids are only physically embedded in the gluten as vesicles (Marion et al., 1987) and do not significantly contribute to its rheological properties (Hargreaves et al., 1995). In line with these latter findings, Gan et al. (1995), Sroan and MacRitchie (2009) and Sroan et al. (2009) provided evidence that in wheat dough, lipids at their natural levels do not notably affect the rheological behavior of the gluten-starch matrix; instead, these authors demonstrated that surface-active components like polar lipids stabilize liquid lamellae surrounding gas cells, thus enabling a larger loaf volume. For zein-starch dough, hydroxypropyl methylcellulose (HPMC) could exert a function similar to polar lipids in wheat dough, stabilizing the gas bubbles, which in turn can be trapped in zein strands (Schober et al., 2008).

Zein has many obvious differences from wheat gluten. There is no protein class analogous to HMW-GS present (Hamaker and Bugusu, 2003; Zhu et al., 2007). Consequently, no large, linear, disulfide-linked polymers are formed. In vivo, γ- and β-zeins are disulfide-linked into networks, forming the outer layer of typical protein bodies (Esen, 1987; Lending et al., 1988), but this is not equivalent to the formation of linear polymers in wheat dough. Commercial zeins are predominantly α-zein (Zhu et al., 2007), and α-zein is typically monomeric, as it is very low in cysteine (Esen, 1987). Size-exclusion chromatography under non-reducing conditions showed predominantly monomers and only some smaller oligomers in maize flour, commercial zein, and in commercial zein aggregated with water at 40 °C to a gluten-like substance (Schober et al., 2008; Zhu et al., 2007). This suggests that zein monomers aggregate via non-covalent bonds into a gluten-like substance capable of forming strands and stabilizing zein-starch dough. Zein has some special properties, mainly its hydrophobicity, which is considerably higher than the hydrophobicity of many other prolamins including wheat prolamin (Belitz et al., 1986; Chiue et al., 1997). Hydrophobic interactions thus need some focus when trying to understand zein-starch dough, and lipids naturally need attention, because they might affect hydrophobic interactions.

In addition to proteins and lipids, the contribution of the starch granules in the dough must not be overlooked. In our previous study (Schober et al., 2008), we found that overly fine zein particles formed only weak zein-starch dough that lacked distinct zein strands. Presumably, the abundant starch granules could keep very small zein particles apart upon mixing, while larger particles could penetrate the surrounding starch barrier and merge with other large zein particles, thus forming fibrils.

Based on the theoretical background introduced in this section, the present study focuses on content and composition of proteins and lipids in zein, on the structure of the zein particles, their aggregation into viscoelastic zein with water at elevated temperatures, the rheological properties of this aggregated, viscoelastic zein and on zein strand formation in zein-starch dough. The main goal remains the identification of those physicochemical differences between commercial zein lots relevant for their baking quality.

2. Experimental

2.1. Materials

Two zein lots (115K0115 and 057K0156) were obtained from Sigma-Aldrich (St. Louis, MO). They are coded Z1 and Z2, respectively, throughout this study. Water content by Karl Fischer was 1.6–1.7% based on the supplier's information. For practical purposes, moisture contents were regarded as sufficiently close to zero to be neglected. Z2 had a distinctly higher crude protein content than Z1 (90.5% versus 84.0%, respectively; N × 6.25, AACC (2000) Method 46-30; Table 1). Particle size distribution of the zeins was measured ($n \geq 2$) with a laser diffraction particle size analyzer (Beckman Coulter LS 13 320, Fullerton, CA); Z2 was overall coarser (Table 1).

Other baking ingredients included unmodified maize starch (11.5% moisture; Bob's Red Mill, Milwaukie, OR), hydroxypropyl methylcellulose (HPMC; Methocel K4 M, food grade, Dow, Midland, MI), table salt, granulated sugar (saccharose), and active dry yeast (purchased locally).

Solvents for defatting (chloroform, hexane) and preparation of HPLC mobile phases were of HPLC grade or otherwise highest available purity. Fluorescein 5(6)-isothiocyanate (FITC) for microscopy was a mixture of two components, for fluorescent labeling of proteins (Sigma–Aldrich).

2.2. Bench-scale defatting

Hexane and chloroform were tested for bench-scale defatting of zein Z1. Preliminary tests suggested that chloroform was more effective. It is, however, the less desirable solvent because of its toxicity. Therefore, hexane was used close to its boiling point to increase its efficiency as an extractant. In detail, the solvents were added to zein (5 ml/g) in a beaker, stirred, covered, and allowed to sit for 1 h at 65 °C (hexane) or room temperature (chloroform). Afterwards, the solvents were removed by decanting, and fresh solvents (5 ml/g) added. This extraction procedure was repeated for a total of three times. After initial removal of the bulk of solvent over night at room temperature in a fume hood, the remaining solvent was removed under vacuum in a freeze-drier over night. Hexane- and chloroform-defatted Z1 are abbreviated Z1, Hexane and Z1, $CHCl_3$, respectively, in this article.

Table 1
Characterization of the raw and bench-scale defatted zeins.[a]

Zein sample	Zein powder						Hydrated, aggregated zein above its T_g		
	Composition[b]			Particle size (μm)			Water binding (%)[e]	Dynamic temperature sweep	
	Protein (%) (N × 6.25)	Lipid (%) (ASE, Hexane)	Lipid (%) (ASE, $CHCl_3$)[c]	10% <	50% <	90%[d] <		Temperature at G'_{min} (°C)[f]	G'_{min} (kPa)[f]
Z1	84.0c	2.50a	7.97a	137a	471b	1215b	61b	64.4a	1.9b
Z1, Hexane	87.5b	0.39d	6.10c	138a	469b	1239b	71a	63.6b	1.8b
Z1, $CHCl_3$	87.5b	0.48c	6.57b	135a	483b	1330ab	75a	62.4c	2.2b
Z2	90.5a	1.59b	3.49d	135a	814a	1567a	79a	58.9d	8.3a
LSD[g]	0.6	0.04	0.23	8	64	265	9	0.7	2.3

[a] Numbers not sharing a common lower-case letter within each column are significantly different ($P < 0.05$).
[b] Moisture of all zeins negligible because of low initial moisture of Z1/Z2 (<2%) and vacuum drying of Z1, Hexane and Z1, $CHCl_3$.
[c] Additional sample (Z2, Soxhlet extracted with $CHCl_3$) included: 0.25% lipid (ASE, $CHCl_3$), significantly lower than all other samples.
[d] Volume %.
[e] Water (%) bound by 1 g zein after mixing (40 °C), pressing (2 kg) and drying of surface.
[f] G'_{min}, minimum of the storage modulus G′ during temperature increase.
[g] Least significant difference.

Additional experiments: (1) a mixture of iso-propanol and hexane (3:1 by volume) was tested with the same protocol as chloroform as a potential replacement. (2) A Soxhlet extractor was used to extract 10 g Z2 with a batch of 500 ml chloroform for 8 h. The defatted zein was kept and residual solvent removed as above.

2.3. Accelerated solvent extraction (ASE)

ASE served as an automated control method to extract the maximum amount of lipids from the bench-scale defatted and undefatted zeins, respectively, using a combination of high temperature (100 °C) and high pressure (69 bar) with both, hexane and chloroform. ASE was conducted with 1–2 g zein as described previously (Moreau and Hicks, 2005), except for the higher temperature and the different solvents. For lipid yield data, the entire hexane or chloroform extracts were dried under a gentle stream of nitrogen at 40 °C, and weighed. Analyses were done in triplicate.

2.4. Baking experiments

The bake test in this study was a modification of a previously described breadmaking procedure (Schober et al., 2008), adapted for the production of round, hearth-type rolls. The basic amount of zein was 40 g Z2. The amount of Z1 and of the defatted zeins was adapted to obtain a protein content equivalent to that in 40 g Z2 (e.g. 43.1 g Z1). The remaining ingredients were 160 g maize starch, 4.0 g HPMC, 10.0 g sugar, 4.0 g table salt, 2.0 g active dry yeast and 150 g water. Dry ingredients were carefully hand-mixed and pre-warmed to 40 °C for >1 h. Then, the water (pre-warmed to 40 °C) was added, and the dough mixed with the help of a Kitchen Aid (Ultra Power, 300 W; St. Joseph, MI) equipped with a dough hook, for 30 s at the lowest level (1 of 10), the bowl scraped and mixing continued at level 2 of 10 for another 30 s. A bulk rest followed (20 min at 40 °C and 80% relative humidity (RH), covered with plastic foil, in a proof cabinet; C5 1, InterMetro, Wilkes-Barre, PA). The dough was then kneaded by hand until smooth, scaled into two 180 g-portions, and each portion was gently rounded by hand into a roll. Subsequently, an intermediate rest was allowed (5 min at 40 °C and 80% RH, covered), and then the two rolls were re-rounded gently, placed on a bake tray, and proofed for 35 min at 40 °C and 80% RH. The rolls were then baked in a deck oven (1T2, Doyon, Linière, Qc, Canada) for 20 min at 225 °C top and bottom heat. Directly before and after loading, steam was injected until condensation occurred visibly on the rolls.

After 60 min of cooling, the rolls were weighed, their volume determined (rapeseed displacement) and their width and height measured. From these data, specific volume [volume (ml)/weight (g)] and width-to-height ratio [width (cm)/height (cm)] were calculated. The values for the two rolls from one batch were averaged into one value, which was treated as one replicate for further statistical analysis. Each baking experiment was replicated at least three times.

In preliminary experiments, only Z1 and Z2 were compared. 40 g of each were used without correction for their different protein content. Rounding was slightly more intense than in the final procedure.

2.5. HPLC analysis of non-polar lipids

Non-polar lipid classes were analyzed via a previously reported normal phase non-polar lipid HPLC method (Moreau, 2006), slightly modified. Dried (nitrogen stream) hexane extracts from ASE were dissolved in hexane (with 0.01% butylated hydroxytoluene, BHT) at a concentration of 1 mg/ml, and 100 μl injected in the HPLC system described by Moreau (2006). In addition to the Charged Aerosol Detector, an Agilent Model 1100 diode array UV detector at 280 nm was also used. Analyses were done in triplicate.

Standard curves of the following non-polar lipid classes were constructed (in the range of 1–20 μg per injection) and the results were used to quantify the amount of each non-polar lipid class: sterol esters were quantified with a cholesterol palmitate standard, free sterols were quantified with a sitosterol standard, ferulate phytosterols were quantified with a γ-oryzanol standard, triglycerides with triolein standard, free fatty acids with separate standards of stearic, palmitic, oleic, linoleic and linolenic acids, diacylglycerols with 1,2 diolein and 1,3 diolein.

2.6. HPLC analysis of polar lipids

Polar lipids were quantitatively analyzed by a modification of a previously described HPLC-ELSD method (Moreau and Hicks, 2005). Dried (nitrogen stream) chloroform extracts from ASE were dissolved in chloroform/methanol (85/15, v/v, with 0.01% BHT) at a concentration of 5–10 mg/ml and 10 μl injected in the HPLC system. *p*-coumaroyl feruloylputrescine (CFP) and diferuloylputrescine (DFP) were both analyzed by UV absorbance at 320 nm using standards prepared in a previous study (Moreau et al., 2001). β-Carotene eluted at 1 min, and lutein and zeaxanthin eluted at 2.5 min and were detected at 450 nm as previously

described (Moreau et al., 2007). The ternary gradient consisted of: solvent A, hexane/acetic acid, 1000/1; solvent B, iso-propanol, and solvent C, water. Gradient timetable (modified relative to Moreau and Hicks, 2005): at 0 min, 90/10/0 (%A/%B/%C); at 20 min, 61/37/2; at 40 min, 12/80/8; at 50 min, 12/80/8; at 51 min, 50/50/0; at 52 min, 90/10/0; and at 60 min, 90/10/0. Analyses were done in triplicate.

Standard curves of the following polar lipid classes were constructed (in the range of 1–20 μg per injection) and the results were used to quantify the amount of each polar lipid class: β-carotene, lutein, zeaxanthin, ferulic acid, *p*-coumaroyl feruloylputrescine (CFP), diferuloylputrescine (DFP), phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and lyso-phosphatidylcholine.

2.7. Size-exclusion (SE) HPLC of proteins

SE-HPLC was conducted in order to compare size distribution of the zeins. For this purpose, zein (5 mg) was extracted for 30 min with continuous shaking, using 1 ml of 12.5 mM Na-borate buffer, pH 10, containing 2% sodium dodecyl sulfate (SDS). Then, the sample was centrifuged ($9300 \times g$, 4 min), 15 μl of the supernatant injected in the SE-HPLC system and analyzed as described previously (Schober et al., 2007).

2.8. Light microscopy with zein particles

Structure and surface properties of zein particles (Z1; Z1, $CHCl_3$) were studied by light microscopy using a digital microscope (VHX-600E, Keyence, Osaka, Japan) equipped with a VH-Z20 zoom lens (20×–200×). Zein particles were stained with Naphthol Blue Black (Amido Black 10B). This is a protein dye, carrying sulfonate, hydroxyl, amino, and nitro groups at an aromatic ring system. It has been suggested that its primary mechanism to bind to proteins are ionic interactions between the sulfonate groups of the dye and basic amino acid residues in the protein (Racusen, 1973; Wilson, 1979). However, the existence of additional bonds, including hydrophobic interactions with proteins, could also be considered (Racusen, 1973). Potential hydrophobic interactions required to verify that Naphthol Blue Black has no notable affinity to hydrophobic phases. Indeed, preliminary tests showed that it was not markedly soluble in hexane or chloroform, and that upon addition of water to these solvents, the dye only stained the water phase.

A Naphthol Blue Black solution in water (0.03 mg/g) was prepared, chilled to ≈1 °C and added to zein (1 ml dye solution for 5 mg zein). After mixing (5 s, Vortex mixer), the sample was centrifuged (2 min, $12{,}000 \times g$), the solution discarded and the pellet (zein particles) washed twice with 1 ml cold water (≈1 °C), respectively, with intermediate decanting. Zein particles were put on a microscope slide, allowed to dry (30 °C) and then examined by light microscopy.

2.9. Aggregation and cohesiveness of zein

The tendency of zein to aggregate in an aqueous environment was tested by short, intense mixing of zein powder and water at 40 °C in the same ratio as in the baking experiments, followed by manual stretching. For this purpose, zein (1 g) was weighed into a screw-capped plastic tube (50 ml), pre-warmed to 40 °C, and 3.75 g water (40 °C) added. This suspension was mixed for 5 s at maximum speed on a Vortex mixer (G-560, Scientific Industries, Bohemia, NY). During this mixing, the different zein samples formed aggregates of varying cohesiveness. These aggregates were removed from the excess water, carefully and slowly manually stretched and photographed. Analyses were done in triplicate.

An additional experiment was done with extensively defatted zein. This was a sample of zein Z2, which was Soxhlet defatted, followed by ASE, using chloroform in both methods (residual lipid <0.25%). Its cohesiveness and extensional properties after mixing with water at 40 °C were evaluated manually.

2.10. Rheology of aggregated zein in sealed cell

A ViscoAnalyser 50 (Reologica Instruments, Lund, Sweden) equipped with a pressurized sealed cell was used in combination with a serrated plate measuring system (20 mm diameter). The pressure in the sealed cell was set to 1 bar (0.1 MPa) above ambient pressure. Increased pressure prevents evaporation of water at elevated temperatures (Madeka and Kokini, 1996).

Fundamental rheology was conducted with fully hydrated, aggregated, viscoelastic zein prepared above its T_g. This is the analogous substance to wet gluten from wheat and has been named 'zein gluten' in a previous study (Schober et al., 2008). However, we now prefer the term 'aggregated zein' to avoid confusion with wheat gluten or corn gluten meal. In contrast to zein-starch dough, in this aggregated zein, starch-starch and starch-protein-interactions cannot affect the rheological results, so that direct conclusions on protein properties are possible (Schober et al., 2008).

For sample preparation, 2 g zein (pre-warmed to 40 °C) and 4 g water (40 °C) were mixed with a spatula. The aggregated zein was removed from excess water, and a portion of 1.7 g weighed out, while at the same time being trimmed to a disk-like shape of ≈2 cm diameter with a razor blade. These 1.7 g aggregated zein samples were compressed with a standard weight (2 kg) for some seconds at 40 °C, the surface dried with a tissue, and the size and shape finally adjusted to a disk of 20 mm diameter by pushing and stretching. This disk was placed centrally between the plates of the rheometer, which had been pre-warmed to 40 °C. After closing of the cell surrounding plates and sample, pressure (1 bar) was applied, the gap adjusted to 2 mm and the sample allowed to relax and equilibrate for 20 min. Subsequently, a creep test directly followed by a dynamic temperature sweep were conducted. The test settings were as follows.

Creep test: creep time 100 s at 200 Pa, followed by 500 s recovery time at 0 Pa, 1 data point per second recorded.

Dynamic oscillatory temperature sweep: 1 Hz, target strain 1×10^{-2}, with a temperature profile (10 min at 40 °C, followed by a linear gradient of 1 °C/min to 95 °C), 1 data point per minute recorded, delay time 10 s, two integration periods. (The target strain of 1×10^{-2} was in the linear viscoelastic region based on stress sweeps.)

All tests were replicated three times within a randomized block design.

2.11. Water binding of aggregated zein

Aggregated zein was prepared as described for the sample preparation for rheology in the sealed cell above. However, only 1 g zein and 2 g water were used at the beginning, and the total amount compressed with the 2 kg-weight and its surface dried. Then, this aggregated zein was weighed (weights were 1.5–1.8 g). Water binding (%) was this weight minus 1 g for the weight of the zein. All tests were replicated three times within a randomized block design.

2.12. Laser scanning confocal microscopy (LSCM) with zein-starch dough

Doughs for LSCM were prepared with essentially the same formulation described for the baking experiments. However, yeast

was omitted, the amounts were downscaled (basis: 1 g zein Z2 and 4 g maize starch), and the fluorescence dye FITC was added to the water used for dough preparation (1 mg/100 ml). The amounts of Z1; Z1, Hexane; and Z1, $CHCl_3$ were corrected to obtain the same protein content as in 1 g Z2 as described for the baking experiments. Doughs were hand-mixed at 40 °C with a spatula, and a small portion smeared in a thin layer on a cover glass. This dough sample on the glass was then covered with immersion oil to prevent drying and placed in a heated (40 °C) clear culture dish system (Delta T3, Bioptechs, Butler, PA). The microscope (Zeiss LSM 5 PASCAL) and laser system have been described previously (Schober et al., 2007). In the present study, the Plan-Neofluar 10×/0.3, and 20×/0.5 objectives were used in combination with an optical slice thickness of 11 and 4 μm, respectively. Between 3 and 11 optical slices of the fluorescence images were projected into one image. In all cases, multiple samples were studied in various positions to be able to select the predominant structures, and thus show representative images.

2.13. Statistics

Unless otherwise stated, completely randomized designs were used. If statistical probabilities are reported, then *F*-tests were followed by pairwise comparisons using Fisher's LSD method ($P < 0.05$). For tests where randomized block designs were used (rheology in sealed cell, water binding of aggregated zein), random block effects were incorporated in the general linear model.

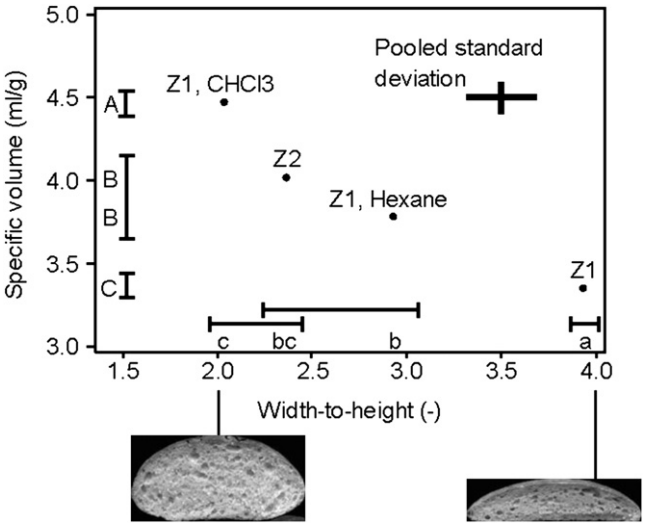

Fig. 1. Volume and shape of rolls produced from zein-starch doughs with raw and bench-scale defatted zeins. Treatments sharing the same lower-case letter along the *x*-axis are not significantly different for the width-to-height ratio ($P > 0.05$), while those sharing the same upper case letter along the *y*-axis are not significantly different for specific volume ($P > 0.05$). [Z1: zein lot no. 1; Z1, Hexane/Z1, $CHCl_3$: Z1, bench-scale defatted with hexane and chloroform, respectively; Z2: zein lot no. 2]. The pictures at the bottom show vertical cuts through rolls with width-to-height ratios of 2.0 and 4.0, respectively, for illustration purposes.

3. Results and discussion

3.1. Baking experiments

Preliminary experiments indicated an unexpectedly distinct difference between two commercial zein lots (coded Z1 and Z2) in their baking performance. Use of Z1 resulted in flat rolls (width-to-height ratio ≈3), use of Z2 resulted in round rolls (width-to-height ratio <2) closely resembling wheat rolls. The amount of zein in the formulation was not corrected for the different protein contents of Z1 and Z2.

The results of the main baking tests are summarized in Fig. 1. As in the preliminary tests, Z1 produced flat rolls with low volume, while rolls from Z2 were significantly rounder and larger in volume. In the main tests, zein levels were corrected to obtain the same protein content in all treatments. The significantly lower protein content of Z1 relative to Z2 (Table 1) therefore cannot be the responsible factor for its inferior performance.

3.2. Effect of lipids

Lipids might affect the shape of rolls by a variety of mechanisms, including, for example, the formation of slip planes in the zein phase (compare Grosskreutz, 1961 for wheat gluten). The inferior zein lot (Z1) was therefore bench-scale defatted. While hexane defatting caused a moderate improvement (Z1, Hexane), chloroform-defatting (Z1, $CHCl_3$) improved volume and shape beyond those values obtained with Z2 (Fig. 1). (The magnitude of the improvement in shape can be estimated with the pictures of rolls at the bottom of the figure.) This result is surprising since bench-scale defatting with either solvent did not reduce the total lipid content, as determined by ASE with chloroform, considerably. It only sank from 8.0 to 6.1–6.6%, while Z2 had a lipid content of only 3.5% (Table 1). ASE extraction with the less polar solvent, hexane, only extracted a maximum of 2.5% lipid (Table 1). Analysis of the lipid composition of the ASE-hexane extract ('non-polar lipids') by HPLC (Table 2) showed that even in this non-polar extract, >10% moderately polar lipids (free fatty acids, diacylglycerols) were present, which might explain the better performance of chloroform as an extractant. Differences between the four zein samples, although significant in several cases, did not seem to provide an explanation for the superior baking performance of Z1, $CHCl_3$, and Z2. Only for total diacylglycerols were Z1, $CHCl_3$, and Z2 very similar and at the same time significantly different from Z1 and Z1, Hexane. This might suggest focusing on diacylglycerols in future studies. However, when considering the minor effect of bench-scale defatting on total lipid content by ASE with chloroform, this finding seems of limited importance for the present study. Analysis of the ASE-chloroform extract ('polar lipids') with a different, polar HPLC system showed similar trends. The components identified were – besides early-eluting non-polar lipids – β-carotene and other carotenes, lutein plus zeaxanthin, ferulic acid, *p*-coumaroyl feruloylputrescine (CFP), diferuloylputrescine (DFP), and lysophosphatidylcholine isomers. For none of these was there a characteristic similarity between Z1, $CHCl_3$, and Z2 combined with a clear difference from the remaining two samples (data not shown). However, Z2 exceeded Z1 in its content of β-carotene and lutein plus zeaxanthin, reflecting the more intense yellow color of Z2 (data not shown). The presence of DFP has been linked to off-odors of zein (Sessa and Palmquist, 2008). In the electronic supplementary material, a typical chromatogram of the 'polar lipids' from Z2 is presented (Online Appendix, Fig. A1).

In summary, bench-scale defatting with chloroform modifies Z1 in a way that appears to exceed the effect that would be caused by a simple, slight decrease in lipid content. The next section addresses a possible modification or denaturation of the protein phase by hexane or chloroform. Before, however, it is worth mentioning that Z1 was additionally bench-scale defatted with an iso-propanol:hexane mixture (3:1), in an attempt to replace the toxic chloroform. The resulting rolls were acceptable (specific volume 4.00 ± 0.05 ml/g; width-to-height 2.5 ± 0.2; average ± standard deviation, $n = 4$). However, chloroform remained the best solvent for defatting (Fig. 1).

Table 2
Lipid composition (% by weight) of the fraction obtained by ASE with hexane ('non-polar lipids').[a]

	Sterol esters[b]	Free sterols[c]	Ferulate phytosterol esters	Triacylglycerols	Total free fatty acids[d]	Total diacylglycerols[e]
Z1	16.9a	1.33b	0.72b	28.9b	8.2a	4.6a
Z1, Hexane	14.6ab	1.15b	0.67b	25.3b	8.0a	4.2a
Z1, $CHCl_3$	17.1a	1.23b	0.51c	19.9c	7.2a	2.9b
Z2	12.5b	2.54a	1.23a	34.2a	7.1a	3.2b
LSD[f]	2.5	0.25	0.06	4.2	1.5	0.6

[a] Numbers not sharing a common lower-case letter within each column are significantly different ($P < 0.05$).
[b] Also includes highly non-polar lipids like wax esters and alkanes.
[c] Sterols + stanols.
[d] Stearic acid + palmitic acid + oleic acid + linoleic acid + linolenic acid + eicosapentanoic acid.
[e] Sum of all identified 1,3 and 1,2 diacylglycerols.
[f] Least significant difference.

3.3. HPLC analyses of proteins

It was hypothesized that defatting with hexane or chloroform might cause a detectable denaturation and resulting change in protein size distribution (e.g. aggregation, visible in SE-HPLC); or different surface hydrophobicities of individual proteins because of refolding and exposure of different side chains (visible in RP-HPLC). However, SE-HPLC indicated no difference between Z1; Z1, Hexane

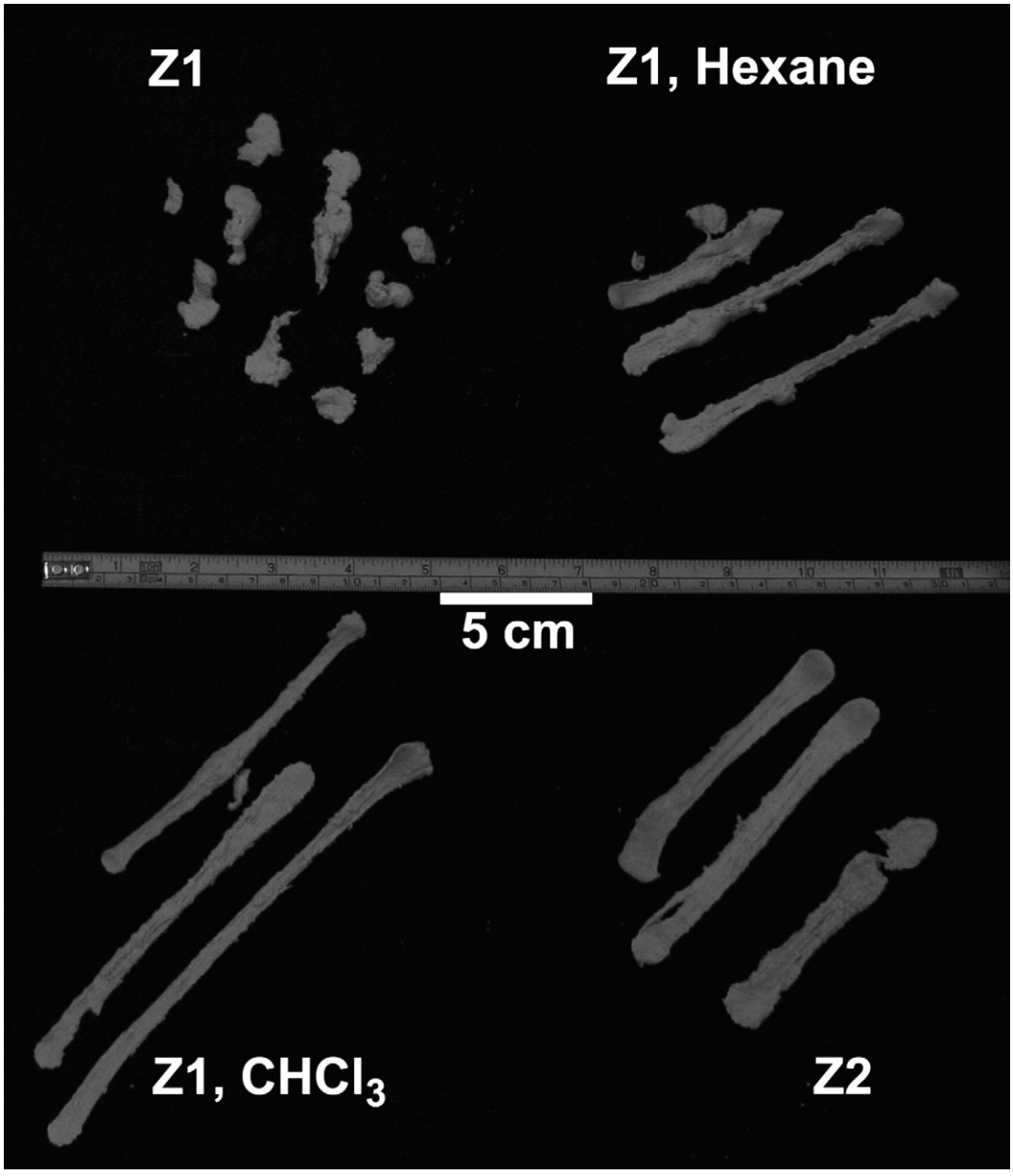

Fig. 2. Zein strands produced by short, intense mixing of zein powder and water above zein's T_g, followed by manual stretching (triplicate analyses).

and Z1, $CHCl_3$ (data not shown). The alkaline SDS-containing buffer used for protein extraction might be sufficiently denaturing by itself to mask any effects of hexane or chloroform. However, SE-HPLC showed that Z2 relative to Z1 had a protein size distribution shifted towards larger sizes (possibly including oligomers) (Online Appendix, Fig. A2). This finding might possibly contribute to the better performance of Z2 relative to Z1.

RP-HPLC also showed no differences between Z1; Z1, Hexane and Z1, $CHCl_3$ (data not shown). This is plausible, because for RP-HPLC, the alkaline SDS-containing protein extraction buffer described for SE-HPLC was used, together with a reducing agent (2% β-mercaptoethanol). A different method is therefore required to test for the effects of bench-scale defatting on Z1. This method has to be sufficiently benign to not mask small changes possibly caused by the defatting.

3.4. Light microscopy of zein particles

As a benign method for examining the effects of defatting on zein particles, light microscopy of particles stained with the protein dye Naphthol Blue Black was conducted. A cold aqueous environment was used to minimize any changes to the zein particles. The undefatted zein Z1 and the bench-scale defatted zein Z1, $CHCl_3$ (worst and best baking performance, respectively) were compared. The color images at two magnifications are shown in the electronic supplementary material (Online Appendix, Fig. A3). The chloroform-defatted zein particles were clearly more intensely stained than the undefatted zein particles. This suggests that zein particles are covered by a lipid film that prevents staining with a more hydrophilic protein dye, unless they have been defatted, preferably with chloroform. A lipid film on the particle surface might also interfere with the aggregation of zein particles into strands above T_g in an aqueous environment, because it might prevent direct interaction of proteins and quick absorption of water. Finally, this hypothesis might also explain that removal of surface lipids on zein particles might have large technological effects despite a small overall reduction of the lipid content (Table 1). In this case, it has to be assumed that the bulk of lipids is located inside the zein particles as layers or vesicle-like bodies, formed upon precipitation of the zein during manufacture. Unlike the surface lipids, they would not be easily extracted unless more radical conditions (like in ASE) are used. The overall surface lipid hypothesis is summarized in a schematic way in the Online Appendix, Fig. A4.

It should be mentioned that in addition to surface-defatting, denaturation of the proteins at the surface of zein particles might also be possible when exposing them to chloroform. If this additional hypothesis should be true, then this denaturation might lead to a change in protein conformation, facilitating protein-interactions (e.g. exposure of hydrophobic domains of the protein facilitating hydrophobic interactions). Currently, however, we lack evidence for this kind of protein denaturation because HPLC analyses of proteins were inconclusive, and the surface lipid hypothesis remains the simplest model that can explain the observed effects.

3.5. Aggregation and cohesiveness of zein

In order to further verify the surface lipid hypothesis, the aggregation of the different zeins in excess water above T_g was compared, applying only a short intense mixing (5 s on a Vortex mixer). After this short mixing step, the aggregated zein was carefully stretched. Undefatted Z1 did not aggregate well and fell apart upon stretching (Fig. 2). As suggested by the surface lipid hypothesis, the zein particles would have difficulty absorbing water, and protein-interactions between the zein particles might be hampered by the thin layer of surface lipids. Hexane seemed capable of partly defatting the particles' surfaces. The strands were, however, still somewhat brittle, visible in their rugged surfaces and limited extensibility (Fig. 2). Chloroform-defatting promoted interaction of zein particles more efficiently, and the resulting strands were more cohesive, extensible and smoother. This suggests that the relevant surface lipids are more polar and not sufficiently soluble in hexane. This observation agrees with the higher efficiency of chloroform relative to hexane to remove lipids by ASE (Table 1). Finally, undefatted Z2 formed strong, cohesive strands. They were less extensible than those from Z1, $CHCl_3$ (Fig. 2). The reason for this needs further research. The overall lower lipid content of Z2 – and thus the lack in slip planes, its higher content in protein oligomers (see above) and also the zein particle size (Table 1) are possible reasons. One additional experiment was conducted, in which Z2 was extensively defatted by Soxhlet method, followed by ASE using chloroform. The resulting zein (<0.25% residual lipid) was cohesive after mixing with water at 40 °C, but was very stiff, firm and only slightly extensible. This supports the hypothesis that lipids inside the zein particles contribute to the extensibility of aggregated zein.

3.6. Rheology with aggregated zein

Examples of curves measured using small-scale dynamic oscillatory measurements in a sealed cell between 40 and ≈70 °C are presented in Fig. 3. The other samples showed the same qualitative behavior. G' reached a clear minimum with increasing temperature and then started to increase, while G'' decreased and then typically leveled out in the given temperature range. A similar behavior of zein at small amplitude oscillation and increasing temperature has been reported by Madeka and Kokini (1996). These authors assumed an initial region of entangled polymer flow, in which both, G' and G'' decreased as temperature increased. The minimum and subsequent increase of G' they attributed to cross-linking reactions, possibly involving disulfide interchange. These authors suggested that for cross-linking, sufficient temperature and moisture are required, so that the zein molecules become mobile and can come together for reaction. However, in addition, hydrophobic interactions should be considered. Bushuk (1998) pointed out that the energy of hydrophobic bonds increases with increasing temperature, possibly providing additional stability during the oven phase. This point appears particularly relevant for zein because of its hydrophobicity.

The temperatures at the minimum of G′ for the different zeins, indicating the onset of cross-linking, are listed in Table 1. They agree

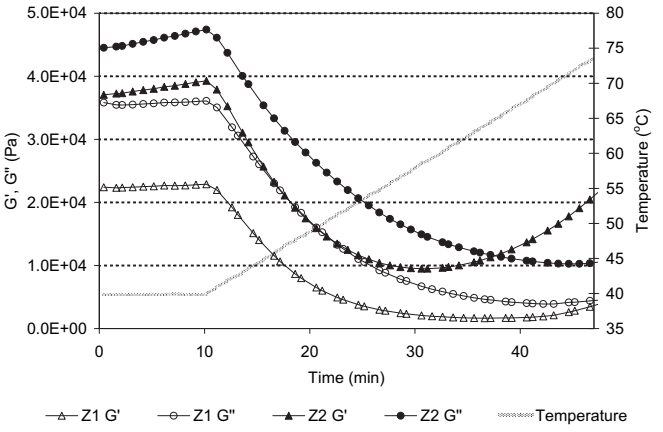

Fig. 3. Examples of dynamic oscillatory temperature sweeps conducted with aggregated zeins (Z1, Z2) in a pressurized sealed cell (G', G'': storage modulus and loss modulus, respectively).

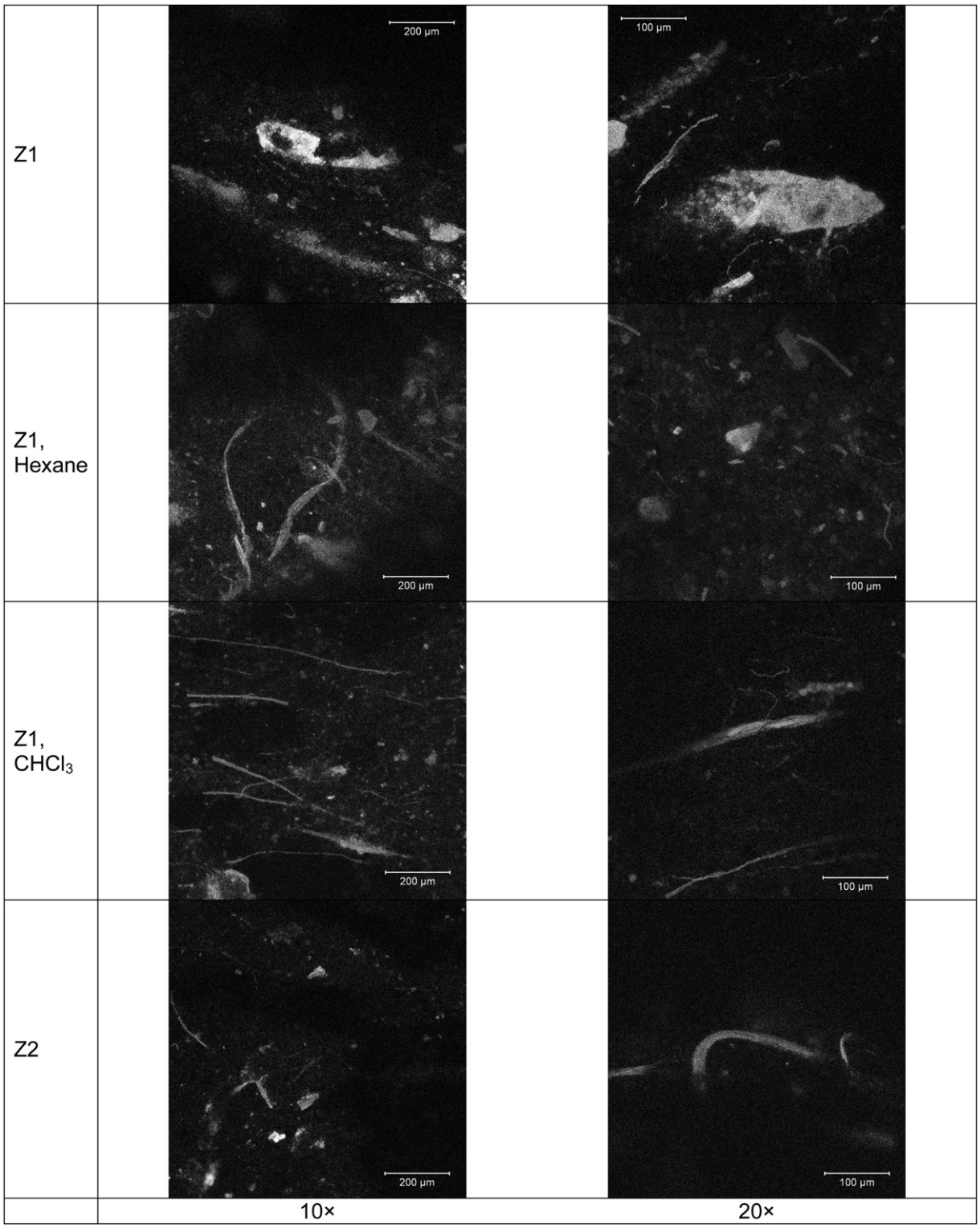

Fig. 4. Laser scanning confocal micrographs of zein-starch doughs prepared from raw and bench-scale defatted zeins. Protein (zein) was specifically stained with FITC. Left side: 10× objective, scalebar 200 µm; right side: 20× objective, scalebar 100 µm.

reasonably with the value of 65 °C, reported by Madeka and Kokini (1996) for fully hydrated zein. Defatting lowered the onset temperature for cross-linking following the order Z1; Z1, Hexane and Z1, $CHCl_3$. For the latter, cross-linking started 2 °C lower than for Z1 (Table 1). This is a further confirmation of the surface lipid hypothesis. The better the zein particles are defatted at their surface, the easier – and thus at lower temperatures – can zein crosslink. Earlier cross-linking would explain that, during baking, the rolls are better stabilized by the cross-linked zein, thus becoming rounder. Z2 stood out insofar as it started cross-linking at the lowest temperatures of all zeins. Table 1 also shows the magnitude of the minimum values of G' (G'_{min}). Z2 had the highest value for G'_{min} (8.3 kPa versus ≈2 kPa for the other zeins). As stated above, the peculiarity of Z2 needs further research.

Defatting increased the water binding. This effect was only significant between Z1 (61%) and the other zeins (≥71%) (Table 1), however, it followed the expected trend. As the total lipid content did not change much upon defatting (Table 1; ASE, $CHCl_3$), a direct competition lipid versus water appears not the likely cause. Instead, better surface-defatting could create a more stable zein network that could better hold the water during the pressing step with the 2-kg weight upon sample preparation.

3.7. LSCM

Representative micrographs of doughs from each zein, obtained by LSCM, are shown in Fig. 4. Differences existed between those zeins in their ability to form strands. Long, extended zein strands were most frequent in dough prepared from Z1, $CHCl_3$. Strands in this sample included thinner and thicker ones, as visible in the higher magnification image (20× objective). In comparison, strands in Z1, Hexane and particularly Z1 were much less frequent and tended to be shorter and less extended. Instead, many blotches of zein were visible, sometimes smeared over a larger area. Dough prepared from zein Z2 takes an intermediate position. It was characterized by less frequent strands than the Z1, $CHCl_3$ sample, and abundant blotches. However, the microscopic strands tended to be thicker, corresponding to the tendency of Z2 to form strong, cohesive, thick strands also on a cm-scale as shown above (Figs. 2 and 4).

Overall, the LSCM results suggest that the aggregation of zein particles to strands is also a critical step in the dough, and that the removal of surface lipids from Z1 by chloroform favored this aggregation.

4. Conclusions

In conclusion, hearth-type rolls resembling wheat rolls can be produced from zein-starch dough. The mechanism of dough formation appears different from regular wheat dough. On a molecular level, large disulfide-linked polymers are absent. On the μm to mm level, the aggregation of zein particles to strands above zein's T_g is a critical step to achieve dough strength. Defatting of the surfaces of the zein particles helps in their aggregation. In addition, particle size of the zein, its protein size on a molecular level and its total lipid content need attention. In future research, emphasis should be put on avoiding toxic solvents like chloroform. The current study suggested that mixtures of solvents like iso-propanol and hexane might be suitable, although more research is needed to find the optimal solvent mixture. In general, this research emphasizes the importance of a multi-scale approach including the molecular, microscopic and macroscopic range, and might also add new aspects to the better understanding of wheat dough.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jcs.2010.07.004.

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