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Localization of Puroindoline-a and Lipids in Bread Dough Using Confocal Scanning Laser Microscopy

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Puroindolines are lipid-binding proteins from wheat flour that play a significant role in bread crumb texture. The localization of wheat flour lipids and puroindoline-a (PIN-a) in bread dough was studied by confocal scanning laser microscopy (CSLM). Wheat lipids were located around gas cells (GC) and embedded within the protein−starch matrix (SPM) of the dough. PIN-a was mainly located in the matrix of dough, where it was associated with lipids. In contrast, in defatted dough, PIN-a was found around GC. Addition of puroindolines in bread dough induced a defatting of the gas bubble surface and a decrease of the lipid vesicles and/or droplet size embedded within the SPM. Therefore, puroindolines control the lipid partitioning within the different phases of dough, a phenomenon that should have important consequence on the gas bubble expansion and GC formation in the further stages (fermentation, baking) of the bread-making process.

KEYWORDS: Confocal microscopy; bread dough; gas bubble; wheat lipid; puroindoline

INTRODUCTION

Bread texture strongly depends on the formation and expansion of gas cells (GC) during the bread-making process. The mechanisms involved in the stabilization of GC are complex and not well understood. Dough is a very dynamic system in which the composition of surface-active molecules, that is, lipids and proteins, covering the gas bubbles could change during the baking process (*1*). Among the surface-active compounds, wheat lipids play a significant role in bread volume (*2*, *3*). This effect is the result of the formation and stability of the dough foam that involves the surface properties of polar lipids and competitive adsorption between lipids and proteins (*4*, *5*). It has been proposed that puroindolines, the lipid-binding proteins of wheat seeds, play a major role in the formation and expansion of the GC in bread dough (*4*, *6*). We have highlighted a close relationship between the fine grain structure of bread crumb and the amount of puroindolines added to flour from a puroindoline-free cultivar (*7*). These effects of puroindolines on bread crumb structure could be due to their interaction with lipids, which determine the resistance of puroindoline foam to the destabilization by lipids (*6*, *8*−*10*).

Two main isoforms with a molecular mass around 13 kDa, puroindoline-a (PIN-a) and puroindoline-b (PIN-b), were isolated from wheat seeds (*11*, *12*). Amino acid sequence analysis has revealed that PIN-a contains 115 amino acid residues with five disulfide bridges and a unique tryptophan-rich domain (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys) (*11*, *12*). The amino acid sequences of PIN-a and PIN-b display ∼60% homology, but the tryptophan-rich domain of PIN-b is truncated (Trp-Pro-Thr-Trp-Trp-Lys). The indole ring of tryptophan is known for its ability to form both hydrogen and hydrophobic bonds with lipids and, therefore, is generally located at the polar−non polar interface of lipid bilayers (*13*−*15*). In the same way, monolayer studies have shown that both PIN-a and PIN-b spread very well and form stable monolayers at the air−water interface (*16*). It has also been found that both PIN-a and PIN-b insert in wheat lipid monolayers (*17*). PIN-a interacts more strongly with lipids than PIN-b and PIN-a is more efficient than PIN-b to prevent destabilization of protein foams by lipids (*6*, *10*).

To get more information on the functionality of wheat lipids and puroindolines in bread-making, we have studied their localization in bread dough by confocal scanning laser microscopy (CSLM). This technique needs minimal sample preparation and provides higher resolution compared to classical fluorescence microscopy. A multilabeling technique was used for the localization of GC, PIN-a, and lipids in bread dough.

MATERIALS AND METHODS

Materials. All of the fluorescent probes were provided by Molecular Probes Inc. (Eugene, OR): Bopidy (665/676) [$C_{29}H_{23}BF_2N_2$, (*E*,*E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene], Rhodol Green (480/521) [Rhodol Green carboxylic acid, hydrochloride (5(6)-CRF 492), $C_{21}H_{14}ClNO_6$], and Alexa Fluor 350 carboxylic acid, succinimidyl ester (346/445) ($C_{16}H_{14}N_2O_9S$). Silicic

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acid was purchased from Fluka (Buchs, Switzerland). Nucleosil C4 300 Å and silica 5 μm 100 Å HPLC columns (0.45 cm × 25 cm) were purchased from CIL (Paris, France) and Merck (Darmstadt, Germany), respectively.

A solution of Bodipy (11.2 mM) in DMSO and a solution of Rhodol Green (2.4 mM) in 10 mM PBS, pH 8.4, were used. Both solutions were stored in the dark at −20 °C. All other chemicals used in our experimental work were of analytical grade. Millipore water was used throughout the experimental work.

Methods. *Extraction of Flour Lipids.* The choice of organic solvents to extract lipids is essential to maintain the functional properties of gluten proteins while allowing extraction of a maximum of total nonstarch wheat lipids (*2*, *18*). From our experience, methylene chloride, more volatile and less toxic than chloroform, provided an excellent compromise. Briefly, wheat flour (500 g) was defatted by vigorous stirring with 1 L of methylene chloride for 1 h at room temperature. The slurry was filtered through a Büchner funnel, and the procedure was repeated twice. The lipid content of non-defatted and defatted flour was determined by gas chromatography after transmethylation of wheat lipids according to the procedure described by Welch (*19*). The defatted flour still contained ∼0.62% lipids (g/g of dm), which should correspond mainly to tightly bound starch lipids.

Phospholipids were extracted from 1 kg of wheat flour with 4 L of methylene chloride/methanol (2:1). The extract was stirred for 2 h at room temperature and filtered on a Büchner funnel. After evaporation of the solvent, the lipid extract was washed according to the procedure described by Folch et al. (*20*). About 0.5 g of lipids in 25 mL of CH_2Cl_2 was loaded on a column (5 cm × 30 cm) packed with 50 g of silicic acid equilibrated in CH_2Cl_2. Lipids were eluted successively with 1 L of CH_2Cl_2 (nonpolar lipids), 1 L of acetone (glycolipids), and 1 L of methanol (phospholipids). Phospholipids were dried under nitrogen, weighed, and finally stored at −20 °C in CH_2Cl_2. The composition of the lipid fractions was determined by normal phase HPLC on a column packed with silica 5 μm 100 Å and a light scattering detection (*21*).

Isolation and Purification of Puroindolines. PIN-a was isolated and purified using Triton X-114 phase partitioning and a chromatographic technique, respectively. The experimental details are described elsewhere (*6*). In reconstitution experiments, a crude puroindoline fraction was used. This fraction was obtained from large-scale cation exchange chromatography and contained >95% puroindolines with ∼80% of PIN-a and ∼20% of PIN-b (*6*).

Fluorescent Labeling of PIN-a. PIN-a was conjugated with the fluorophore Alexa Fluor 350 carboxylic acid, succinimidyl ester, as described by the manufacturer (Molecular Probes). Briefly, 10 mg of PIN-a was solubilized in 2 mL of 0.1 M sodium bicarbonate buffer, pH 8.3. The Alexa Fluor 350 succinimidyl ester was dissolved in DMSO at 10 mg/mL just before the start of the reaction. Fifty microliters of the reactive dye solution was slowly added to the protein solution, and the reaction was incubated in the dark for 1 h at room temperature with continuous stirring. The reaction was stopped by adding 0.1 mL of freshly prepared 1.5 M hydroxylamine, pH 8.5. The hydroxylamine was incubated for 1 h at room temperature to remove unstable dye conjugates. Labeled PIN-a was separated from unreacted reagent by extensive dialysis against distilled water, and this separation was followed by spectrofluorometry. The purity of the Al-PIN-a was checked by C4 reversed phase HPLC. The efficiency of the labeling reaction was determined by measuring the absorbance of the protein at 280 nm (A_{280}) and the absorbance of the dye (A_{346}) at its maximum wavelength (λ_{max} = 346 nm).

The protein concentration of the Alexa Fluor 350 protein conjugate and the degree of labeling were calculated from the following equations as described by the manufacturers:

$$\text{protein concn (M)} = \frac{A_{280}[-(A_{346} \times 0.19)]}{\epsilon_{\text{protein}}} \times \text{dilution factor} \quad (1)$$

ϵ is the molar extinction coefficient of PIN-a, and 0.19 is a correction factor to account for absorption of the dye at 280 nm.

The degree of labeling was calculated from

$$\text{mol of dye/mol of protein} = \frac{A_{346} \times \text{dilution factor}}{19000 \times \text{protein concn (M)}} \quad (2)$$

where 19000 is the approximate molar extinction coefficient of the Alexa Fluor 350 dye at 346 nm.

Surface Tension Measurement. The surface activity of unlabeled PIN-a and Al-PIN-a was checked from surface tension measurement using a Kruss digital tensiometer (K10) interfaced with a computer for automatic data recording. The experimental details were described elsewhere (*22*).

Incorporation of Fluorescent Probes in the Dough. The fluorescent probes were incorporated by mixing 2 g of wheat flour, 44 mg of NaCl, 1.2 mL of distilled water, and the required amount of fluorescent probes. For labeling of lipids, 112 nmol of Bodipy was added to the ingredients; for labeling of the starch−protein matrix (SPM), 24.3 nmol of Rhodol Green was added; and for localization of PIN-a, 1 mg of Al-PIN-a was added to the ingredients. All of the doughs were prepared with the same moisture content (60%). Ingredients were mixed in the bowl of a mixograph at 88 rpm for 10 min, and the doughs were stored at −20 °C. This mode of incorporation of fluorescent probes into the dough has been already described by Lindsay et al. (*23*).

Addition of Total Puroindolines and Phospholipids into the Dough. An increasing quantity of total puroindolines was added to the basic ingredients of dough, with levels of 0.1, 0.2, 0.5, and 1% (g/g wt of flour) in the presence of lipid fluorescent probes (0.11 μM Bodipy) and of SPM fluorescent probes (24.3 nM Rhodol Green).

Phospholipids were dispersed by vortexing 20 mg of phospholipids in 300 μL of distilled water for 1 min. The phospholipid dispersion was then added to the other basic ingredients of the dough in the presence of lipid and SPM fluorescent probes. The quantity of added wheat phospholipids accounted for 1% (g/g) of the weight of flour. The same experiment was performed by dispersing 20 mg of phospholipids in the presence of 3.5 mg of total puroindolines in 300 μL of distilled water. The phospholipid−puroindoline mixture was then added to the basic ingredients of the dough in the presence of Bodipy and Rhodol Green. The concentrations of phospholipids and puroindolines were 1 and 0.15% g/g of the flour, respectively, on a dry matter basis.

Confocal Laser Scanning Microscopy (CLSM). The doughs containing fluorescent probes were observed with a Zeiss LSM 410 Axiovert microscope. This microscope was equipped with four lasers, a helium−neon laser, which emitted a monochromatic ray at 633 nm, a helium−neon laser, which emitted a monochromatic ray at 543 nm, a blue argon ion laser, which emitted a monochromatic ray at 488 nm (blue), and a UV argon ion laser, which emitted a line at 363.8 nm. CLSM was used with the appropriate laser excitation and band-pass (BP) filters for each fluorescent probe. Al-PIN-a was excited by the 363.8 nm line of the UV argon ion laser with a long pass (LP) at 397 nm. Rhodol Green was excited by the 488 nm line of the blue argon ion laser with a BP of 515−565 nm. Bodipy, which was a long-wavelength fluorescent dye, was excited by the 633 nm line of the helium−neon laser with an LP at 665 nm. After thawing, a small dough sample was deposited on a slide and covered by a cover glass. This system prevented the sample from drying during the observation. Blends were examined with a water immersed ×40 objective. Images were obtained serially by scanning the bread dough piece with the corresponding laser beam in combination with an appropriate emission filter for each fluorescent probe. Each image was recorded separately in different channels (R, G, B). Overlaying of the recorded images permitted simultaneous visualization of distinct structures. There was an average of eight images in each series.

RESULTS

The aim of this study was to colocalize gas cells, lipids, and PIN-a in bread dough. Therefore, we developed a multilabeling technique (MLT), which consisted of introducing three different fluorescent probes in the bread dough sample, Bodipy, Rhodol Green, and Alexa Fluor 350 labeled PIN-a. Alexa Fluor 350 is quite soluble in water, and its fluorescence is nonsensitive to pH in the 4−10 range (*24*). The surface activity of Al-PINa

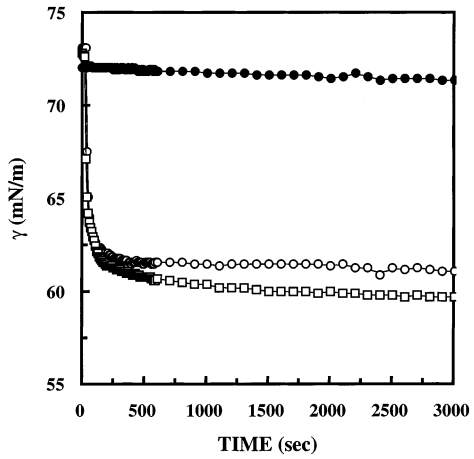

Figure 1. Surface tension measurements of PIN-a and Al-PIN-a at the air–water interface (protein concentration = 1.6 μg/mL in 100 mM NaCl and at 25 °C: (●) mol of Alexa 350/mol of PIN-a, (1/3) $n = 3$; (○) mol of Alexa 350/mol of PIN-a (1/1), $n = 1$; (□) unlabeled PIN-a.

depended on the extent of labeling. For one molecule of Alexa 350 bound per molecule of PIN-a ($r = 1$), the surface properties of Al-PIN-a and PIN-a were identical (**Figure 1**). For $r = 3$, Al-PIN-a had very low surface activities in comparison with the native PIN-a (**Figure 1**). Rhodol Green has greater photostability than fluorescein and exhibits a fluorescence with a low pH sensitivity (*25*). When a triple labeling was performed, the dough sample was observed successively with the red line of the He−Ne laser (Em = 633; LP = 665), the blue line of the argon ion laser (Em = 488; BP = 515−565), and finally the UV laser (Em = 363.8 nm; LP = 397 nm). No interference was detected in the dough between the signals emitted by Bodipy, Rhodol Green, and Alexa 350. Single labeling was automatically done with each probe to check on eventual interference between the three probes during the multilabeling experiments. No fluorescence was observed in the absence of fluorescent probes except in UV excitation, where some fragments of the aleurone layer were detected in very small amount.

Single Labeling of Lipids in Bread Dough. A CLSM image of labeled lipids in bread dough is presented in **Figure 2a**. Bodipy is a hydrophobic fluorescent probe with a high affinity for lipid, oil, and other nonpolar liquid phases. This fluorescent probe gave rise to a dispersion of white spots throughout the dough (see L in **Figure 2a**). Some of these white spots formed a circular alignment (see arrow in **Figure 2a**), which could correspond to the localization of lipids around a GC (20 μm in diameter).

Single Labeling of PIN-a. A laser confocal image of the dough containing Al-PIN-a is presented in **Figure 2b$_1$**. The image of the same sample observed from non-confocal transmitted light microscopy is presented in **Figure 2b$_2$**; both the starch granules (SG) and the GC are clearly visible in the transmitted light image. Al-PIN-a was dispersed in the SPM without specific localization around the GC (**Figure 2b$_1$**). The same experiment was performed by adding Al-PIN-a to the partially defatted flour. The laser confocal image of the dough obtained from defatted flour and Al-PINa is presented in **Figure 2c$_1$**, and the corresponding image obtained with transmitted light is shown in **Figure 2-c$_2$**. **Figure 2c$_1$** shows a more specific localization of Al-PINa around the GC (see arrow in **Figure 2-c$_2$**). It was also observed that all of the Al-PINa was not only localized around the GC but was also in the protein portion of the SPM.

Single Labeling of the Starch—Protein Matrix. The fluorescent labeling of the SPM of the dough was necessary to observe GC in the dough and to have a more precise localization of lipids and PIN-a in the two differents compartments of the dough (i.e., GC and SPM) with the multilabeling system. The laser confocal image of dough containing Rhodol Green is presented in **Figure 2d**. From **Figure 2d**, one could note that when Rhodol Green was added to the dough, the SPM of the dough was totally stained except the GC, which were not fluorescent. GC appeared as black holes in the fluorescent SPM. Rhodol Green is an aqueous fluorophore, which was dispersed throughout the dough except in the GC. Thus, the small GC could be distinguished from the fluorescent SG, which could display the same size at the end of mixing, that is, 5−10 μm (**Figure 2d**).

Double Labeling of Lipids and the Starch—Protein Matrix. Simultaneous observation of different fluorescent probes was possible by superimposing images obtained in the same confocal plane. To study the distribution of lipids in the dough and especially around the bubbles, Rhodol Green and Bodipy were added simultaneously into the dough. First, the dough sample was excited by the red laser ($\lambda_{exc} = 633$ nm; LP = 665 nm). Then, the dough sample was excited by the blue argon ion laser ($\lambda_{exc} = 488$ nm; BP = 515−565 nm). Finally, the two images were superimposed in the channel RGB (**Figure 3a**). The labeled lipids (L) had a green fluorescence around the GC and also in yellow spots, dispersed in the red SPM (the superimposition of green and red gave rise to the yellow color). The same experiment was performed by adding Rhodol Green and Bodipy in partially defatted dough (**Figure 3b**). No green fluorescent labeling was observed around the GC. On the other hand, a low fluorescent signal was highlighted in the SPM. This signal could be due to the labeling of residual lipids (rL) bound to the SPM.

Double Labeling of PIN-a and the Starch—Protein Matrix. To study more precisely the distribution of PIN-a in the dough and especially around the GC, Rhodol Green and Al-PIN-a were added simultaneously to the dough. The dough sample was excited by the blue argon ion laser ($\lambda_{exc} = 488$ nm; BP = 515−565 nm). It was then excited with the UV laser ($\lambda_{exc} = 363.8$ nm; LP = 397 nm). After superimposition in channel RGB, the final image was obtained (**Figure 3c**). GC formed black holes in the red SPM dough. Al-PIN-a had a yellow fluorescence (superimposition of green and red fluorescence) in the red SPM. The same experiment was performed by adding Rhodol Green and Al-PIN-a to a partially defatted dough (**Figure 3d**). The Al-PIN-a appeared yellow in the red SPM but in green around the GC.

Triple Labeling of Lipids, PIN-a, and the Starch—Protein Matrix. The dough image containing Bodipy, Rhodol Green, and Al-PINa is presented in **Figure 3e$_3$**. By this triple labeling, it was possible to study the localization of PIN-a and lipids in the SPM by superimposing images obtained successively from excitation by the red, blue argon, and UV lasers (**Figure 3e$_3$**). The superimposition of green and red gave rise to a yellow color for the lipids in the SPM (**Figure 3e$_1$**), the superimposition of red and blue gave rise to the magenta color for Al-PIN-a in the SPM (**Figure 3e$_2$**), and, finally, the superimposition of the three images gave rise to a white labeling for lipids and Al-PIN-a colocalized in the SPM (**Figure 3e$_3$**).

Effect of Puroindolines on the Localization of Lipids in the Dough. Total puroindolines were added to wheat flour at 0, 0.1, 0.5, and 1% (g/g of dry wheat flour), and the corresponding CSLM images obtained from double labeling (lipids

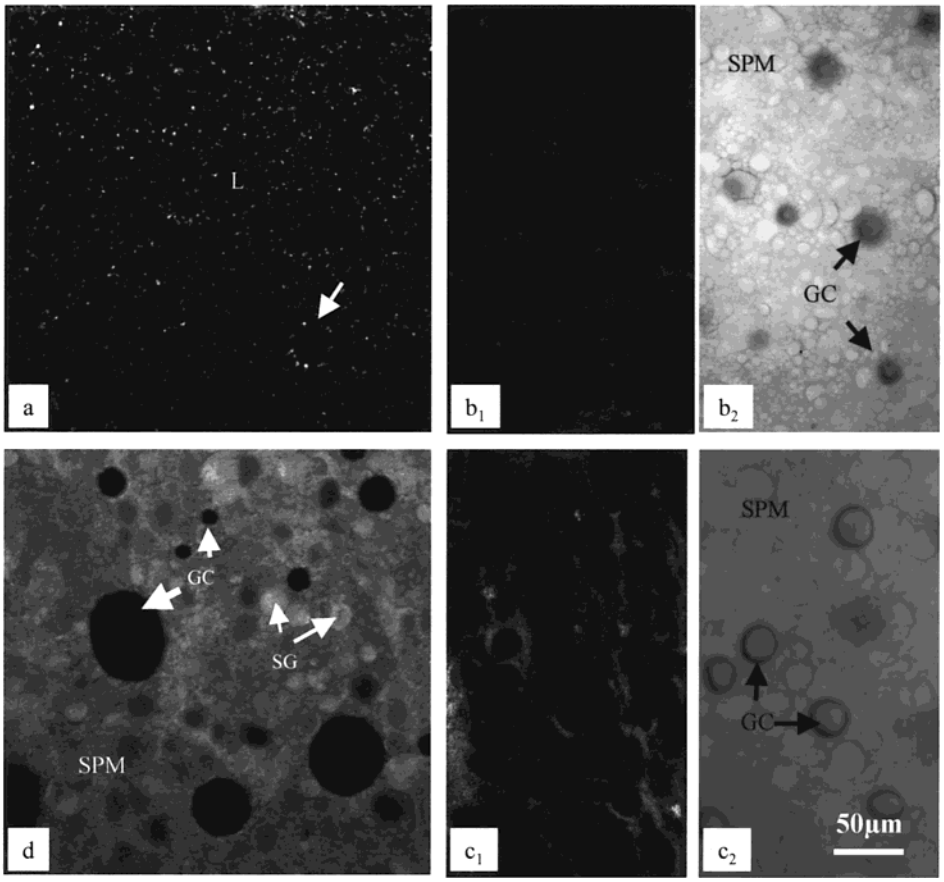

Figure 2. (a) Laser confocal image of dough containing Bodipy. Lipids appeared as bright white spots dispersed in the entire sample. White spots are also shown around a bubble (arrow). **(**b_1) Laser confocal image of dough containing Al-PIN-a. The fluorescent signal of Al-PIN-a was dispersed in the SPM. (b_2) Same image of dough containing Al-PIN-a, observed with transmitted light microscope. This image highlights the position of the GC in the sample. (c_1) Laser confocal image of defatted dough containing Al-PIN-a. (c_2) Same image of defatted dough containing Al-PIN-a obtained with transmitted light microscope in which it was possible to see GC. (d) Laser confocal image of dough containing Rhodol Green. All of the SPM of the dough was fluorescent except GC. It was easy to highlight GC and SG. The scale bar is the same for all figures, bar = 50 μm.

and SPM) are presented in **Figure 4a**−**d**. Lipids (L), which were located around the GC appeared green, and lipids (L) dispersed in the SPM were yellow stained. When the total PIN content increased, most of the labeling of lipids around the GC disappeared and lipids were mainly dispersed in the SPM, where they formed small spots.

Effect of Puroindolines on the Localization of Exogenous Wheat Phospholipids. In a first experiment wheat phospholipids (1% g/g of dry wheat flour) were added to the dough as described under Materials and Methods. The corresponding CSLM image obtained after double labeling of lipids and the SPM is presented in **Figure 4e**. Lipids (L) were localized around the GC and dispersed as green and yellow spots in the matrix. A second experiment consisted of mixing phospholipids (1% g/g of dry wheat flour) with total puroindolines (0.15% g/g of dry wheat flour) before addition to the basic ingredients of the dough. The corresponding CSLM image is presented in **Figure 4f**. In the presence of added puroindolines, green lipid spots (L) dispersed in the SPM and localized around the GC appeared smaller in size than in the absence of added puroindolines.

DISCUSSION

The objective of the present work was to localize the most important surface-active molecules, lipids and PIN-a, in bread dough. By using CSLM, we have observed distinctly the nonfluorescent GC in the fluorescent SPM. This image resolution is essential to localize and to understand the role of lipids and PIN-a in the two compartments of bread dough. Therefore, we have shown that at the end of mixing, lipids were localized in the SPM and around the GC. The presence of lipids at the air−dough interface is in agreement with numerous studies in relation to the role of lipids in baking. MacRitchie (*2*, *3*) has proposed a hypothesis in which lipids influence baking performance by affecting foam stability. This theory is supported by reconstitution experiments, which have shown the effect of individual lipid classes on loaf volume. The model of lipid functionality through foam stabilization is supported by several experiments based on lipid behavior in pure aqueous systems. For instance, Carlson et al. (*26*, *27*) have shown that wheat glycolipids form a pure lipid monolayer at a gaseous interface at equilibrium. Paternotte et al. (*28*) have demonstrated that in spread monogalactosyldiglyceride (MGDG)−gliadin films, MGDG was able to remove gliadin from the interface under compression, suggesting that polar lipids will finally cover the gas−liquid interface. A labeled anti-gliadin monoclonal antibody was previously used to locate storage proteins in bread, and no labeling of the GC surface was observed by TEM (*29*). This suggests that gliadins and probably other gluten proteins are not adsorbed on the surface of gas bubbles. Keller et al. (*30*) have suggested that lipids were the major constituents in the GC interfacial film, because lipids are the most surface-active components of the dough. This hypothesis was based on experiments performed on pure protein monolayers to which different lipid suspensions were subsequently added (*30*). They

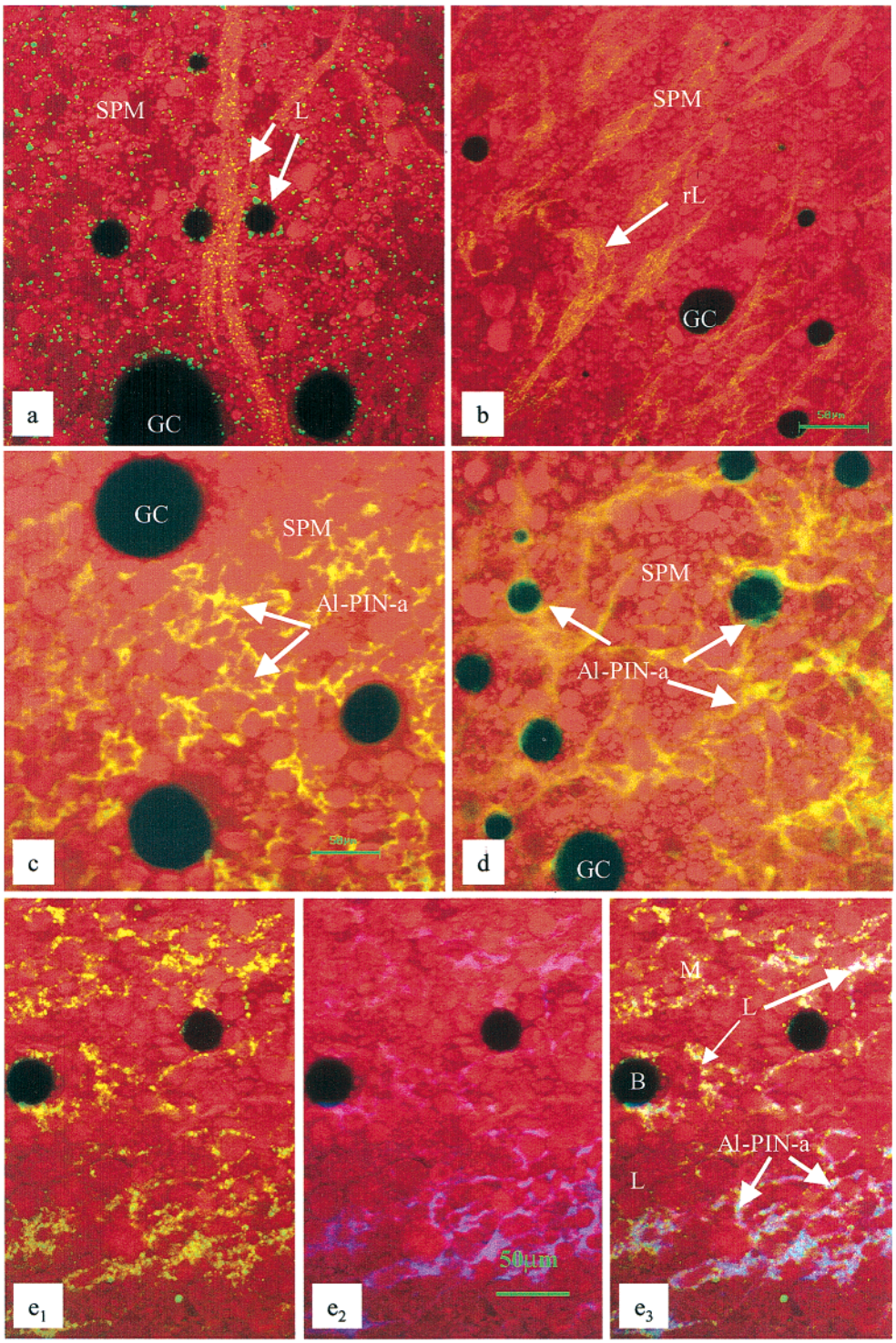

Figure 3. (a) Superimposition of two images obtained from dough containing Bodipy (lipophilic probe) and Rhodol Green (hydrophilic probe). Lipids (L) appeared green and yellow (superimposition of red and green), SPM appeared red, and GC were not fluorescent. (b) Superimposition of two images obtained from "defatted dough" containing Bodipy and Rhodol green. SPM appeared red, GC were not fluorescent, and residual bound lipids (rL) were stained yellow in matrix (superimposition of red and green gives yellow color). (c) Superimposition of two images obtained from dough containing Al-PIN-a and Rhodol Green. Al-PIN-a had a yellow fluorescence in the red SPM (the superimposition of green and red give the yellow color). (d) Superimposition of two images obtained from defatted dough containing Al-PIN-a and Rhodol Green. The Al-PIN-a appeared yellow (superimposition of green and red) in the red matrix (SPM) but also green around the GC. (e_1) Localization of lipid (green/yellow) in the SPM (red). (e_2) Localization of Al-PIN-a (magenta) in the SPM (red). (e_3) Superimposition of e_1 and e_2. The three colors superimposed give the white (colocalization of lipids and Al-PIN-a in the matrix). The scale bar is the same for all figures, bar = 50 μm.

concluded that surface pressures reached by the lipid (≥45 mN/m) are such that protein layers should collapse, resulting in an almost total replacement of proteins by lipids. From our in situ localization studies, we observe that lipids do not cover all of the surface of the GC at the end of mixing. This suggests that other surface-active components should participate in the stabilization of the interfacial film. Eliasson and Larsson (*31*) proposed that the GC are initially stabilized by proteins, followed

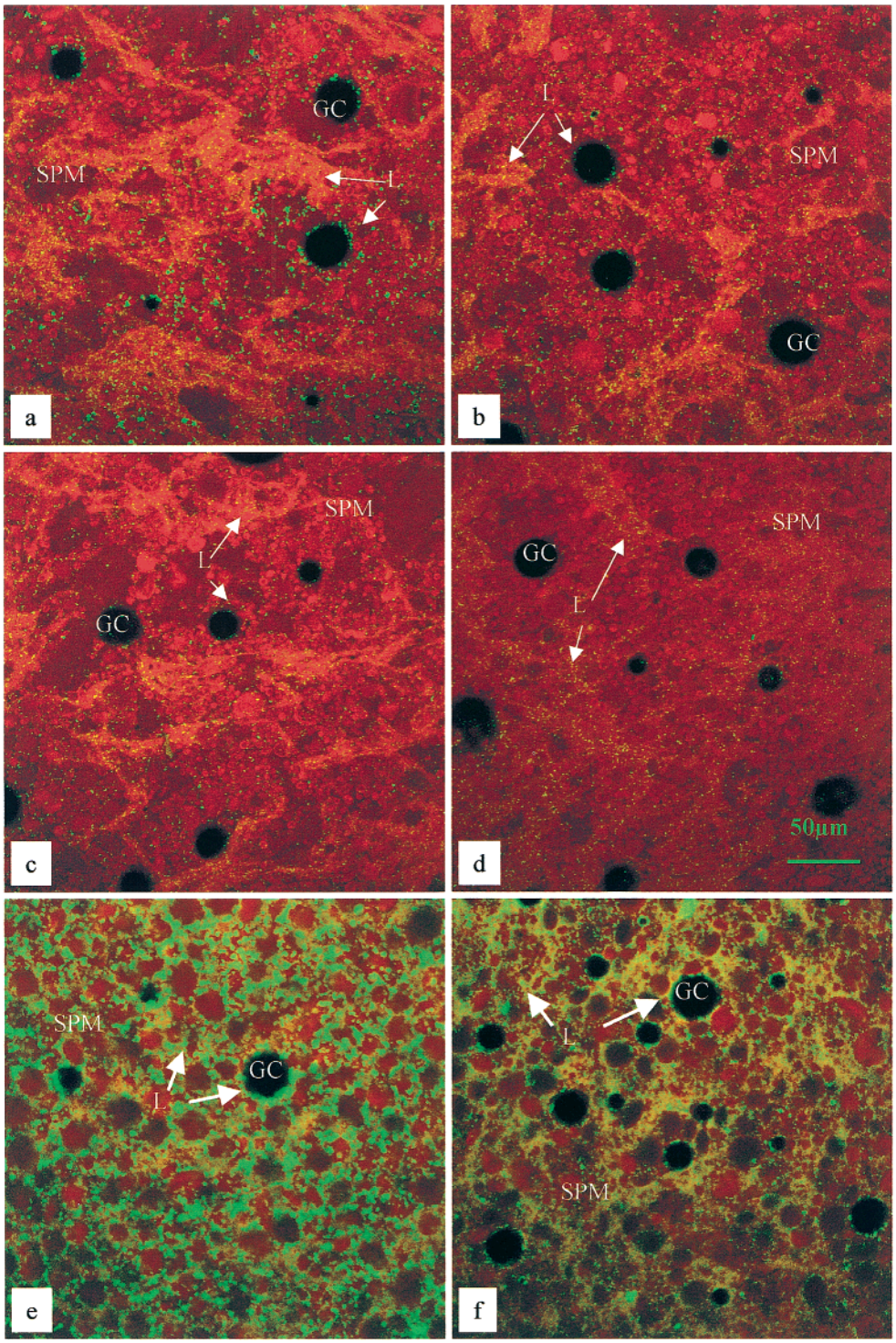

Figure 4. (a–d) Superimposition of two images obtained from dough containing Bodipy (lipophilic probe) and Rhodol Green (hydrophilic probe). Laser confocal images a–d represent, respectively, dough containing 0, 0.1, 0.5, and 1% of added PINs (g/g weight of flour). The observation of the dough has been done in the same conditions as the double labeling of lipids and SPM. Lipids (L) located around the GC appeared green, and lipids (L) dispersed in the SPM were yellow-stained. (e) Superimposition of two images obtained from dough containing Bodipy and Rhodol Green. Dough was enriched with 1% of phospholipids (g/g weight of flour), observed in the conditions of double labeling of lipids and SPM. Lipids (L) localized around the GC and around SG as well as dispersed in the SPM appeared as big green or yellow vesicles. (f) Superimposition of two images obtained from dough containing Bodipy and Rhodol Green. Dough was enriched with a premixture of phospholipids and PINs observed in the conditions of double labeling of lipids and SPM. In the presence of PINs, green lipid globules (L) dispersed in the SPM and localized around the GC appeared smaller than in absence of PINs. The scale bar is the same for all figures, bar = 50 μm.

by lipids during oven spring. This does not agree with our experiments, in which wheat lipids are observed at the bubble interface in the first stage of the bread-making process, that is, the mixing stage. Furthermore, our experiments performed on defatted dough have shown that GC can be stabilized in the absence of lipids and suggest that the interfacial film might be composed of other surface-active components. These results are in accordance with the reconstitution experiments on defatted

flour made by MacRitchie (*32*). He has shown that lipids were not essential to obtain expanded dough and that the dough made from defatted flour has a dispersion of many small and uniformly sized cells at the end of proofing. Because dough prepared from defatted flours can expand and can give bread with a high volume, Gan et al. (*4*) suggested that proteins and/or pentosans dissolved in the dough aqueous phase can take the place of lipids in defatted flour.

Our experiments of localization, which have also shown that lipids were dispersed as oil bodies and/or vesicles into the SPM of the dough, are in good agreement with previous results on wheat gluten and doughs (*33*, *34*). PIN-a was also located in the SPM. The triple labeling of lipids, PIN-a, and the SPM indicates a close localization of lipids and PIN-a in the SPM. This is in agreement with previous observations showing that oil-rich vesicles entrapped in dough are mainly composed of proteins with a molecular mass similar to that of puroindolines (*35*). These results are also in agreement with the capacity of puroindolines to interact spontaneously and strongly with lipid vesicles (*6*). The role of puroindolines in bread-making has been investigated by performing reconstitution experiments (*7*). Addition of 0.1% of puroindolines (g/g) to flour drastically modified both the rheological properties of the dough and the structure of the bread crumb. This effect of puroindolines on bread crumb texture was related to the good foaming properties of puroindolines, especially in the presence of lipids (*6*). Our present experiments do not show a specific localization of PIN-a around the GC except in defatted dough, whereas both PIN-a and PIN-b spread very well at the air−water interface (*17*, *22*) and display good foaming properties of puroindolines (*6*, *10*). On the contrary, the major part of PIN-a was colocalized with lipids in the SPM of dough. This is not surprising if we keep in mind the localization of puroindolines in starchy endosperm (*36*), and if we take into account the strong interaction of puroindolines with lipids (*6*). To support this idea, it is worth noting that oil bodies are triglyceride droplets covered by a monolayer of polar lipids (*37*) in which puroindolines can insert (*17*). Therefore, more than foaming properties, the lipid binding properties of puroindolines can have important consequences on bread crumb texture by preventing the rupture of thin films and bubble coalescence by adsorption of lipid vesicles and/or oil droplets at the interface of foam lamellae (*5*). Therefore, puroindolines behave as defatting agents (detergent effect) in agreement with the fine and regular bread grain crumb obtained in the presence of puroindolines (*7*) and in bread made with defatted flour (*32*). For defatted dough, PIN-a is localized at the surface of gas bubbles. This suggests that, in defatted dough, most of the PIN-a, being not bound by lipids, is available to adsorb on the surface of gas bubbles. Because PIN-a forms foams with fine bubbles, this mechanism agrees with the fine crumb structure of bread obtained from defatted flours (*7*, *32*). Therefore, the foaming and "defatting" properties of puroindolines can lead to bread with fine crumb structure.

We have shown that the addition of increasing quantities of puroindolines has important consequences on the lipid distribution in dough. For instance, when 1% of puroindolines was added to the dough, no lipids were detected at the air−water interface. When an excess of phospholipids (1% g/g) was added to the wheat flour in the form of an aqueous liposomal dispersion, lipids were bothlocalized around the gas bubble and dispersed in the SPM. The addition of puroindoline to the added phospholipids, at a ratio similar to the ratio normally found in a standard bread wheat flour, has a drastic effect on the dispersion of these phospholipids. In the presence of puroindolines, lipid vesicles appeared to be much smaller than in the absence of puroindolines. Therefore, puroindolines could favor the formation of small liposomes in agreement with the detergent effect suggested above. Simultaneously, fewer lipids were observed at the surface of gas bubbles, in agreement with the defatting effect observed when increasing amounts of puroindolines were added to wheat flours.

In conclusion, it appears that puroindolines prevent adsorption of wheat lipids to air−water interfaces, opening the possibility for other surface active components, especially soluble proteins, to form a stable film around gas bubbles. This detergent-defatting effect of puroindolines prevents gas bubbles from coalescence and film rupture by lipid aggregates. Therefore, addition of puroindolines led to a homogeneous size distribution of GC in wheat doughs, similar to what it is observed for solvent-defatted wheat flour. When an excess of puroindolines is added to wheat doughs, lipid-free proteins are available to stabilize these air−water interfaces. Because puroindolines form foams with small bubbles (*6*), this leads also to a homogeneous dispersion of small bubbles in bread doughs in agreement with the fine crumb structure observed with added puroindolines (*7*). Finally, it is important to keep in mind here that the experiments were performed on nonyeasted mixed doughs so that our conclusions are mainly concerned with the initiation of gas bubbles in a dough system. For example, it is not excluded that, on fermentation and baking, an adsorption and spreading of puroindolines−lipid complexes at the air−water interfaces could occur. Further investigations are needed to understand the evolution of gas bubbles and formation of gas cells along the bread-making process.

ABBREVIATIONS USED

PIN-a, puroindoline-a; PIN-b, puroindoline-b; Al-PIN-a, Alexa Fluor 350 labeled puroindoline-a; CLSM, confocal laser scanning microscopy; MLT, multicolor labeling technique; UV, ultraviolet; Em, emission; LP, long pass; BP, band-pass; DMSO, dimethyl sulfoxide; GC, gas cells; SPM, starch−protein matrix; SG, starch granules.

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