Interaction of Puroindolines with Wheat Flour Polar Lipids Determines Their Foaming Properties

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The interaction of puroindolines with wheat polar lipids and the stability of the corresponding puroindoline foams were investigated. Whereas puroindoline-a is capable of binding tightly to both wheat phospholipids and glycolipids, puroindoline-b interacts tightly only with negatively charged phospholipids and forms loose lipoprotein complexes with glycolipids. Both ionic, hydrogen, and hydrophobic bonds contribute to the stability of puroindoline−polar lipid complexes, and the integrity of tryptophan-rich domain is essential for the interaction with neutral polar lipids. Compared with egg white proteins, chosen as a model of nonlipid binding and good foaming food proteins, puroindolines exhibit excellent foam stability, especially in the presence of wheat polar lipids. The higher efficiency of puroindoline-a than puroindoline-b to prevent foam destabilization by wheat polar lipids highlights the close relationships between lipid binding and foaming properties of these wheat proteins. These results indicate that puroindolines would be good candidates to play a major role in the formation and stability of bread dough foams.

Keywords: *Puroindoline; wheat; glycolipids; phospholipids; foam*

INTRODUCTION

Two major basic and cystine-rich lipid binding proteins have recently been isolated from wheat endosperm by Triton X114 phase partitioning (Blochet et al., 1991; Blochet et al., 1993). Amino acid sequencing of the main isoform showed that its polypeptide chain contains 115 residues and five disulfide bridges for a molecular weight of 12 479. A unique tryptophan-rich domain (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys) was present, which led us to name this protein puroindoline-a (PIN-a), from puros, the Greek word for wheat, and indoline for the indole ring of tryptophan. The second isoform was named puroindoline-b (PIN-b), and its primary structure was deduced from the corresponding cDNA sequencing (Gautier et al., 1994). PIN-a and PIN-b exhibit ~60% homology in their sequence but the tryptophan-rich domain of PIN-b is truncated (Trp-Pro-Thr-Trp-Trp-Lys). It has been shown that puroindolines are strictly identical to basic friabilins (Morris et al., 1994) found at the surface of the starch granule, proteins that have previously been suspected to control wheat hardness/softness (Greenwell and Schofield, 1986; Jolly et al., 1993). In fact, most of the friabilins/puroindolines are not bound to starch, suggesting that they could be only a genetic marker of hardness/softness (Jolly et al., 1993). In agreement with their lipid binding properties, starch−puroindoline interactions could be mediated by residual polar lipids found at the surface of purified starch granule (Greenblatt et al., 1994).

Finally, these proteins have intrinsically good foaming properties and their lipid binding properties confer to puroindolines the capability of preventing the lipid-induced destabilization of protein foams (Wilde et al., 1993; Clark et al., 1994; Husband et al., 1995). These unique surface properties of puroindolines make them attractive in breadmaking technology where it has been shown that a close relationship exists between the foaming properties of the aqueous phase of dough and the bread volume. In particular, a positive effect of wheat polar lipids has been shown on both the foaming properties of aqueous wheat flour extracts and bread volumes (see MacRitchie, 1983, for a review). As a component of wheat flour, it has been suggested that puroindolines in association with wheat polar lipids could play a major role in the formation and expansion of the gas cells in bread dough and therefore, on the structure and texture of the bread crumb (Marion and Clark, 1996). Until now, the lipid binding properties of puroindolines have been investigated in the presence of water-soluble lysophosphatidylcholines (lysoPC; Wilde et al., 1993; Husband et al., 1995). Such lipoprotein models composed of lipids forming micelles are far from dough systems where polar lipids are organized in uni- and multilamellar liposomes (Carlson et al., 1978; Marion et al., 1987, 1989). The purpose of this work was to study the relationship between the binding properties of puroindolines to wheat polar lipids and the foaming properties of the puroindoline−lipid mixtures to probe the potential of these proteins in breadmaking technology.

MATERIALS AND METHODS

Materials. Analytical grade solvents were from SDS (Peypin, France). Egg white protein (Ovomousse M251) was kindly provided by Epi Bretagne (Plaintel, France). Diphenylhexatriene was from Sigma-Aldrich (L'Isle d'Abeau, France). Other chemicals were from Merck (Darmstadt, Germany). Silicic acid and Triton X114 were purchased from Fluka (Buchs, Switzerland). Carboxymethylcellulose CM 52 was from Whatman (Millipore, Bedford, MA). Neobar CS cation exchange column was from Dyno particles (Norway). Nucleosil C4 300Å and silica 5 μm 100 Å columns (0.45 × 25 cm) were purchased from C.I.L. (Paris, France) and Merck (Darmstadt, Germany), respectively. Other chromatographic resins, columns, and fast-performance liquid chromatography (FPLC) materials were from Pharmacia (Uppsala, Sweden).

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Purification of Puroindolines. Puroindolines were purified according to a modification of previous procedures (Blochet et al., 1993; Wilde et al., 1993). Five kilograms of dry wheat seeds (*Triticum aestivum* L. cv. Etoile de Choisy) were ground and extracted with 10 L of 0.1 M Tris-HCl pH 7.8 buffer containing 5 mM EDTA, 0.1 M KCl, and 4% Triton X114. The mixture was gently stirred overnight at 4 °C to avoid foaming. After centrifugation for 30 min at 5000*g*, the supernatant was heated at 30 °C for 1 h and centrifuged for 15 min at 5000*g*. The upper detergent poor phase was discarded and the same volume of fresh Tris buffer without TX114 was added. The solution was stirred for 1 h at 4 °C, and the phase-partitioning procedure was repeated. Finally, the upper phase was discarded, the lower detergent-rich phase was removed, and the proteins were precipitated overnight with 10 L of peroxide-free diethyl ether:ethanol (1:3 v/v) at −20 °C. After centrifugation at 2000*g*, the protein pellet was washed twice with the diethyl ether:ethanol mixture and finally, twice with pure diethyl ether at −20 °C. The protein sediment was dried overnight under reduced pressure. Recent studies have shown that such a treatment does not impair the secondary structure and conformation of the puroindolines (Le Bihan et al., 1996).

The dry white protein powder was solubilized in 5 mM MES (2[*N*-morpholino]ethanesulfonic acid buffer, pH 5.5) and was dialyzed overnight at 4 °C against the same buffer. Insoluble material was removed by centrifugation at 10 000*g* for 30 min, and the supernatant was loaded on a column (10 × 100 cm) packed with Sephadex G75. Each fraction was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to the procedure of Laemmli (1970) and fractions containing polypeptides with apparent molecular weights of <20 000 were pooled and loaded on a column (5 × 30 cm) packed with a cation-exchange resin (Whatman CM92). The fractions were eluted by applying a gradient from 0 to 0.7 M NaCl in 8 mM MES pH 5.5 buffer. PIN-a and PIN-b were detected in eluting fractions by C4 reversed-phase HPLC (Blochet et al., 1991). PIN-a- and PIN-b-enriched fractions were pooled, dialyzed overnight against deionized water, and freeze-dried. PIN-a and PIN-b were purified from the puroindoline-enriched fractions by FPLC, and the purity of PIN-a and PIN-b was checked by both SDS−PAGE and reversed-phase HPLC (Blochet et al., 1991, 1993).

Extraction and Purification of Wheat Polar Lipids. Wheat lipids were extracted from 2 kg of wheat flour with 10 L of methylene chloride:methanol (2:1). The extract was stirred for 2 h at room temperature and then filtered through a Buchner funnel. After evaporation of the solvent, the lipid extract was washed according to the procedure described by Folch et al., (1957). About 1 g of total lipids in 50 mL of CH_2Cl_2 was loaded on a column (5 × 30 cm) packed with 50 g of silicic acid in CH_2Cl_2 and were eluted successively with 2 L of CH_2Cl_2 (nonpolar lipids), 2 L of acetone (glycolipids), and 1 L of methanol (phospholipids). In another run, the column was eluted successively with CH_2Cl_2 and methanol to get total nonpolar lipids and polar lipids (glycolipids + phospholipids). Total polar lipids, glycolipids, and phospholipids were dried under nitrogen, weighed, and finally stored at −20 °C as concentrated solution in CH_2Cl_2 (∼100 mg mL^{-1}). Their composition was determined by normal-phase (silica 5 μm 100 Å) and light-scattering detection (Stolyhwo et al., 1987).

Preparation of Aqueous Dispersions of Wheat Polar Lipids. For foaming and lipid binding studies, aliquots of concentrated lipid solutions were poured in glass test tubes, and dried under a nitrogen flow and reduced pressure. Dried lipids were vigorously dispersed by vortexing in 10 mM sodium phosphate buffer, pH 7. After mixing for 5 min, homogenous and milky aqueous dispersions were obtained with phospholipids and total polar lipids. For glycolipids, addition of a few milliliters of peroxide-free diethyl ether to the aqueous solution was necessary to obtain good dispersions. Diethyl ether was gently evaporated under a nitrogen flow.

Intrinsic Fluorescence of Puroindolines. Fluorescence emission spectra of puroindolines were recorded with a spectrofluorometer (SLM 4800, Aminco). Excitation and emission wavelengths were set at 295 and 335 nm (slits 4 nm), respectively. For the titration of puroindolines with various lipids, the following procedure was used: 2−4 μL of aqueous dispersions of wheat total polar lipids, glycolipids and phospholipids in phosphate buffer (0.01 M, pH 7; 1 and 10 mg mL^{-1}) were added in a stepwise manner to 1 mL of puroindoline solution (0.1 mg mL^{-1}, 8 μM) in the same buffer. After incubation for 1 min, the fluorescence emission spectra were recorded at 20 °C. Fluorescence data were corrected for background signals with the corresponding lipid solutions alone. Absorption coefficients of 1.94 mL mg^{-1} cm^{-1} at 280 nm for PIN-a and of 1.66 mL mg^{-1} cm^{-1} at 280 nm for PIN-b were used to determine protein concentration.

Steady-State Fluorescence Polarization. The effect of PIN-a and PIN-b on lipid dynamic was studied by fluorescence polarization of a lipophilic probe, diphenylhexatriene (DPH) embedded within the hydrophobic core of lipid bilayers. Fluorescence intensity was measured with a fluorescence polarization instrument (SLM 4800, Aminco). Excitation and emission wavelengths were set at 360 and 435 nm (bandwidth 4 nm), respectively. Measurements of the fluorescence intensities detected through polarizers oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the plane of polarization of the excitation light beam allowed calculation of the steady-state polarization parameter $P = I_{\parallel} - I_{\perp}/I_{\parallel} + I_{\perp}$.

For DPH labeling, 50 μL of DPH solution (17 μg/mL^{-1}) diluted in methylene chloride were added to 500 μL of the lipid solution (1 mg mL^{-1}, DPH:lipid ratio, ≃1/250). After drying, 1.5 mL of 10 mM sodium phosphate buffer (pH 7) were added and mixed as already described. Then, 1 mL of a diluted solution in phosphate buffer was poured in a quartz cuvette and titration was performed by adding in a stepwise manner a puroindoline solution (1 mg mL^{-1}) in phosphate buffer. The polarization data were recorded at 20 °C.

Foaming Measurements. To analyze the foaming properties of puroindolines, the apparatus previously described by Loisel et al. (1993) was used. Eight milliliters of protein and protein-lipid solutions were poured into a reservoir ending by a column (2 x 20 cm), and foams were produced by sparging solutions with air at a constant flow rate (15 mL min^{-1}) through a porous disk (porosity = 2 μm; radius = 2 cm). Conductivity was determined with a pair of platine electrodes located at the bottom of the column. Gas sparging continued until 35 mL of foam was reached. This fixed foam volume was detected by a camera. When bubbling stopped, the drainage kinetic was recorded by conductivity measurements. The conductivity was related to the volume of liquid sustained by foam within the lamellae (Guillerme et al., 1993).

During these experiments, the protein concentration was constant and equal to 0.3 mg mL^{-1} and the molar lipid/protein ratio varied from 0 to 10. This concentration was chosen because it gives rise to similar foam stability for both puroindolines. Foam stability was characterized by its foam density (FD) after 5 min of drainage according to the relation FD = V_l/V_f, where V_l is the volume of liquid in the foam determined by conductivity measurements and V_f is the volume of the foam determined by the camera.

RESULTS

Lipid Binding. The composition of the wheat phospholipid and glycolipid fractions is given in Table 1. Phospholipids were mainly composed of the *N*-acyl derivatives of phosphatidylethanolamine (NAPE and NALPE). These lipids are negatively charged at neutral pH contrary to the neutral zwitterionic PE and PC. Glycolipids were mainly composed of neutral galactolipids (MGDG and DGDG). The composition of the total polar lipids was not determined because overlapping occured in the elution of phospholipids and glycolipids with our HPLC gradient procedure (results not shown). However, fractionation by silicic acid adsorption chromatography and weighing of dried lipids showed that total polar lipids are composed of 25% phospholipids and 75% glycolipids. This lipid composition is in agreement with previous results on the composition and distribu-

Table 1. Composition of Wheat Glycolipids and Phospholipids[a]

glycolipid	dry weight (%)[b]	phospholipid	dry weight (%)[b]
SG + ASG	15.0	NAPE	40.2
acyl-MGDG	2.1	NALPE	22.7
MGDG	15.9	PE	7.0
MGMG	1.8	PI+PA	11.0
DGDG + DGMG	65.2	PC	18.5
		lysoPC	0.7

[a] (SG +ASG): glycosylated sterol and acyl glycosylated sterol; (MGDG) monogalactosyldiglycerides; (DGDG +DGMG) digalactosyldiglycerides + digalactosylmonoglycerides; (NAPE) *N*-acylphosphatidylethanolamine; (NALPE) *N*-acyllysophosphatidylethanolamine; (PE) phosphatidylethanolamine; (PI + PA) phosphatidylinositol + phosphatidic acid); (PC) phosphatidylcholine; (lysoPC) lysophosphatidylcholine. [b] These values are the average of two determinations.

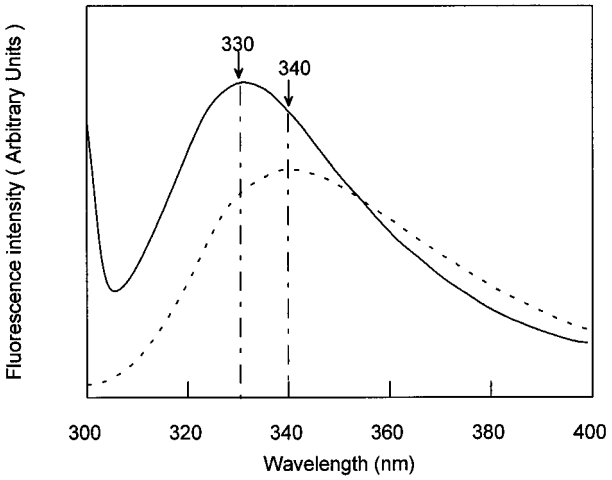

Figure 1. Fluorescence spectra of PIN-b alone (– – –) and PIN-b with wheat total polar lipids (—; R_i = 40) in 10 mM sodium phosphate buffer (pH 7). The wavelength of the maximum of fluorescence emission is indicated by (↓).

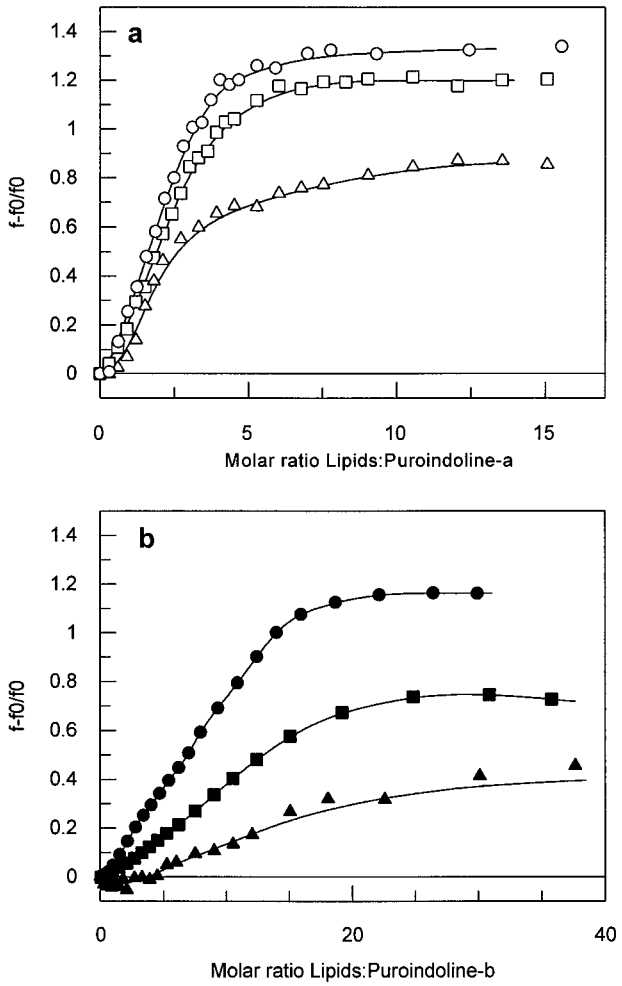

Figure 2. Fluorescence titration curves of (a) PIN-a by total polar lipids (□), phospholipids (○), and glycolipids (△), and (b) PIN-b by total polar lipids (■), phospholipids (●), and glycolipids (▲).

tion of lipids in wheat kernels (Hargin and Morrison, 1980). From these data and from the fatty acid composition of lipid classes provided by Morrison (1988), we have determined a mean molecular weight of 800 for phospholipids and a mean molecular weight of 820 for glycolipids to determine the molar lipid concentrations.

The different wheat polar lipid fractions dispersed in water gave rise to homogenous milky and stable suspensions in agreement with previous studies showing that such lipids form lamellar liquid-crystalline (LC) phases (i.e., liposomes) in aqueous media (Carlson et al., 1978; Larrson and Puang-Ngern, 1979).

The binding of wheat polar lipids to puroindoline enhanced tryptophan fluorescence intensity along with a blue shift of the maximum emission wavelength (Figure 1). At saturation and for all lipids, a blue shift of tryptophan fluorescence of puroindolines from λ_{max} = 340 nm to λ_{max} = 330 nm was observed. This 10 nm blue shift means that tryptophan is in a more hydrophobic environment when lipids are bound to puroindolines (Lakowicz, 1983). Such a shift is generally observed on lipid binding and can be due either to a conformational change of the protein or to a direct interaction of tryptophan residues with acyl chains (Dufourcq and Faucon, 1977; Dufourcq and Faucon, 1978). The fluorescence emission with a λ_{max} centered on 340 nm suggests that tryptophans are not fully exposed to aqueous solvent because, in our conditions, λ_{max} of *N*-acetyltryptophanamide (NATA) in water was 350 nm and in dioxan was 325 nm, in agreement with previously reported data (Lakowicz, 1983). However, it is important to keep in mind that the tryptophan proximity in the sequence of puroindolines can favor stacking of indole rings and generate specific fluorescence properties of tryptophan residues. Finally, PIN-a and PIN-b exhibited similar fluorescence characteristics, although PIN-b has a truncated tryptophan-rich domain and another tryptophan residue far from this domain (Gautier et al., 1994).

At lipid saturation, the maximum fluorescence intensity of PIN-a was higher in the presence of phospholipids and total polar lipids than with glycolipids. For PIN-b, the maximum fluorescence intensity at lipid saturation was in the order phospholipids > polar lipids > glycolipids. At the plateau, maximum fluorescence intensity was lower for PIN-b than for PIN-a (Figure 2).

Sigmoïdal and hyperbolic fluorescence titration curves were obtained according to the polar lipid-puroindoline binary mixture (Figure 2). Therefore, lipid binding to puroindolines could occur either in a noncooperative or in a cooperative manner. The dissociation constant (K_d) and the number of lipid binding sites (n) was determined by fitting experimental curves with lipid binding mathematical models. Therefore, the existence of an equilibrium between free protein (P) in solution, the free lipid site in the lipid bilayer (S) and the protein−lipid complex (PS) was considered.

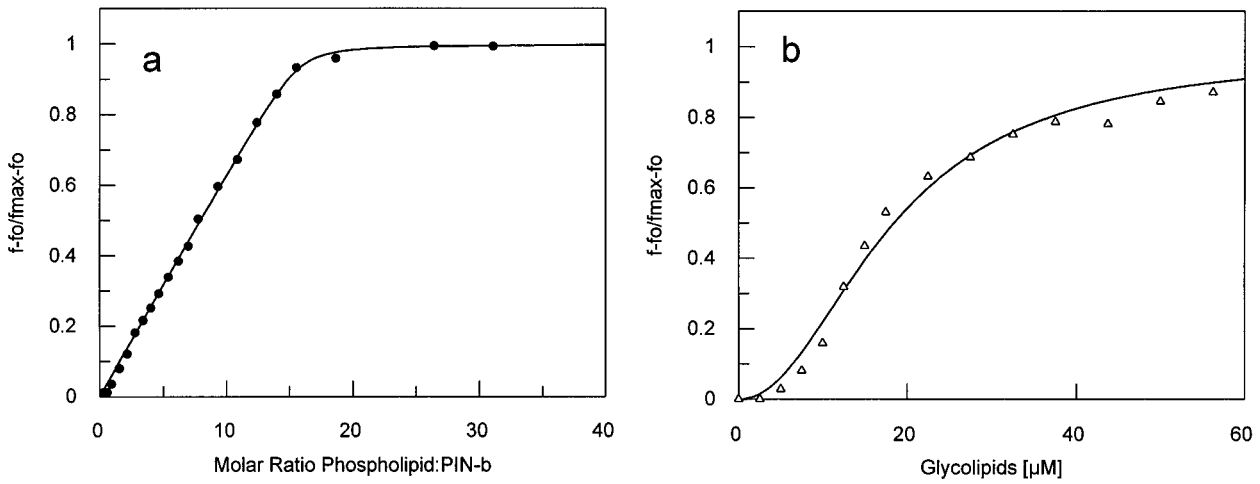

Figure 3. Fitting of fluorescence titration data (—) considering (a) identical and independent binding sites [PIN-b with wheat phospholipids (●)], and (b) cooperative binding using the Hill equation [PIN-a with wheat glycolipids (Δ)].

For the noncooperative model, independent and identical sites were assumed:

$$P + S \Leftrightarrow PS$$

The value of K_d was determined by

$$K_d = (P)(S)/(PS) \quad (1)$$

The bound protein fraction, α, was deduced from the fluorescence intensity measurements as $F - F_o/F_{max} - F_o$, where F_{max} and F_o are fluorescence intensities of bound and free protein, respectively. At equilibrium

$$(P) = (P_o)(1 - \alpha) \quad (2)$$

and

$$(S) = (S_o) - \alpha(P_o) \quad (3)$$

Considering that each site consisted of n molecules of lipids

$$(S_o) = \frac{(L_o)}{(n)} \quad (4)$$

and

$$(S) = (L_o)/n - \alpha(P_o) \quad (5)$$

where P_o is the protein concentration and L_o is the total lipid concentration. Therefore, by using eqs 2, 5, and $R_i = L_o/P_o$, eq 1 is transformed to the second-order equation

$$\alpha^2 - \left(1 + \frac{R_i}{n} + \frac{K_d}{(P_o)}\right)\alpha + \frac{R_i}{n} = 0 \quad (6)$$

with only one solution because $\alpha < 1$ (Dousseau et al., 1986)

$$\alpha = \frac{\left(1 + \frac{R_i}{n} + \frac{K_d}{(P_o)}\right) - \sqrt{\left(\left(1 + \frac{R_i}{n} + \frac{K_d}{(P_o)}\right)^2 - 4\frac{R_i}{n}\right)}}{2} \quad (7)$$

Theoretical curves were calculated using K_d and n corresponding to the best fit as estimated by Newton-Gauss analysis of the experimental values with equation 7 (Figure 3a). This binding model can be applied to the interaction of PIN-a with total wheat polar lipids and phospholipids and returned K_d values in the 10^{-7} M range with four independent and identical lipid binding sites (Table 2). For PIN-b only the interaction with phospholipids can be fitted with such a binding model and returned K_d and n values of ~4 10^{-8} M and 16, respectively (Table 2).

With the other lipids, sigmoïdal shapes of fluorescence titration data suggested a cooperative lipid binding process that was strengthened by an absence of good fitting with the previous model. On the contrary, the experimental data were well fitted with the Hill equation (Hill, 1910), as previously described (Wilde et al., 1993, Figure 3b). The K_d could be deduced by fitting fluorescence data with the equation

$$\alpha = \frac{([L]/K_d)^{Nh}}{(1 + ([L]/K_d)^{Nh})} \quad (8)$$

where α is the fraction of bound protein and Nh was the coefficient of cooperativity. Since n was unknown, the total lipid (L_o), instead of free lipid concentration was used.

The Hill fitting returned a K_d of ~18 μM and a Nh of ~2 for the titration of PIN-a with glycolipids (Table 2). Values of K_d of ~90 and ~105 μM were found in the binding of PIN-b with total polar lipids and glycolipids, respectively (Table 2). The corresponding Hill numbers (Nh) were 2 and 3, respectively (Table 2). However, it was not possible to determine the number of lipid binding sites by only fluorescence titration. Fluorescence titration curves saturated at $R_i = 4$ for PIN-a and glycolipids and at $R_i = 20$ and $= 25$ for PIN-b with polar lipids and glycolipids, respectively (Figure 2). This result suggests that PIN-a has four binding sites for glycolipids, whereas PIN-b has >16 (determined with the noncooperative model for phospholipids) binding sites for glycolipids and total polar lipids.

Steady-State Fluorescence Polarization. The effect of puroindolines on the dynamic of lipid acyl chain was followed by fluorescence polarization of a hydrophobic probe, diphenylhexatriene (DPH), embedded within the hydrophobic core of lipid bilayer. This probe is often used to probe membrane fluidity changes because it partitions in the hydrophobic core of membranes, both in mobile and nonmobile part of lipid

Table 2. Dissociation Constants (K_d), Number of Lipid Binding Sites (n), and Hill Number (Nh) for the Interaction of Puroindolines with Wheat Phospholipids, Glycolipids, and Total Polar Lipids

	PIN-a			PIN-b		
interactant	K_d	n	Nh	K_d	n	Nh
phospholipids	0.14 ± 0.05	4.1 ± 0.1		0.04 ± 0.01	16.3 ± 0.2	
glycolipids	18.2 ± 0.8	nd[a]	2.1 ± 0.2	104.7 ± 7.9	nd	3.1 ± 0.6
total polar lipids	0.16 ± 0.04	4.5 ± 0.1		89.0 ± 5.3	nd	2.0 ± 0.1

[a] nd, not detected.

Table 3. Fluorescence Polarization of DPH Embedded within Wheat Polar Lipids (P_o) and within Puroindoline−Wheat Polar Lipid Complexes Dispersed in Aqueous Phosphate Buffer (P_f at Saturation)

	PIN-a			PIN-b	
compound	P_o	P_f	$(P_f - P_o)/P_o$ (%)	P_f	$(P_f - P_o)/P_o$ (%)
phospholipids	0.142 ± 0.003	0.196 ± 0.003	38	0.182 ± 0.001	28
glycolipids	0.201 ± 0.001	0.233 ± 0.003	16	0.234 ± 0.002	16
polar lipids	0.186 ± 0.006	0.233 ± 0.001	25	0.224 ± 0.001	20

bilayers, does not appear to bind to proteins in the presence of lipids, and has an intense fluorescence (Pink, 1989).

In the absence of puroindolines, the initial polarizations of DPH were between 0.14 and 0.20. These values indicated that lipids were in a fluid state and not in a gel state ($p = 0.5$) in agreement with the high content of polyunsaturated fatty acids in wheat lipids (Hargin and Morisson, 1980). However, it is interesting to note that the fluidity of glycolipids was significantly lower than the fluidity of phospholipids whereas their fatty acid composition exhibits similar high contents in polyunsaturated fatty acids (Clayton and Morisson, 1972). This result could be due to the presence of glycosylated sterols in the glycolipid fraction (Table 1) because sterols are known to decrease lipid motion in the fluid state (Demel and De Kruijff, 1976). Both PIN-a and PIN-b induced an increase of DPH polarization, which means that lipid motion was impaired by the presence of the proteins. This increase of lipid order in the fluid state is generally observed for proteins penetrating in the hydrophobic core of lipid bilayers (Dufourcq et al., 1982). The effect of puroindolines on the increase of DPH polarization is in the order phospholipids > total polar lipids > glycolipids. Furthermore, PIN-b was less efficient than PIN-a in increasing lipid order in phospholipids and total polar lipids. In contrast with phospholipids, both puroindoline isoforms had a low impact on the polarization of DPH embedded within wheat glycolipids (Table 3).

Foaming Properties. None of the lipid fractions used in this work gave rise to 35 mL of stable foam. At the lipid concentration corresponding to $R_i = 10$, 35 mL of foam was obtained, but drainage occurred in a few seconds when gas sparging stopped. This result means that wheat polar lipids adsorb at the air−water interface but do not form stable films in the concentration range used in this study. The concentration of puroindolines (0.3 mg mL^{-1}) was chosen because the densities of PIN-a and PIN-b lipid free foams are similar after 5 min of drainage. In the presence of lipids, PIN-a foams had a higher foam density after 5 min of drainage than PIN-b foams (Figure 4). With total polar lipids, anenhancement of PIN-a foam stability until $R_i = 0.6$ was observed (Figure 4c). This enhancement was lower than that we have previously reported with lysoPC (Wilde et al., 1993). In the case of PIN-a−phospholipids mixtures, no major changes were observed on the foam stability until a molar ratio equal to 1 (Figure 4a), and with PIN-a−glycolipids mixtures, a slight decrease occured from $R_i = 0.1$ to $R_i = 1$ (Figure 4b). Above this ratio, the foam stability of PIN-a−lipid mixtures decreased below the value obtained for PIN-a alone. Phospholipids reduced the stability of PIN-b foams from $R_i = 0.2$ to $R_i = 10$ (Figure 4a). Phospholipid−PIN-b foams, although less stable than phospholipid−PIN-a foams, were more resistant to destabilization than those formed with glycolipids (Figure 5) and total wheat polar lipids. For these polar lipid mixtures, a dramatic negative effect on PIN-b foam stability was observed (Figures 4b, 4c, and 5).

We compared foam density of protein egg white solutions with PIN-a and b foam density, both after 5 min of drainage. In the case of puroindolines, 0.3 mg mL^{-1} was sufficient to obtain a foam density equal to 0.028 after 5 min of drainage, whereas 1.25 mg mL^{-1} of egg white protein was necessary to obtain a close density value (0.023) (Figure 6). A great destabilizing effect of wheat polar lipids on egg white protein foams was observed.

DISCUSSION

In this study, puroindolines interacted with highly aggregated lipid structures and not with simple ligands in solution in contrast with previous studies where micellar monoacyl lysoPC were used (Wilde et al., 1993; Husband et al., 1995). This situation mimics that found in bread dough where polar lipids are organized in uni- and multilamellar LC vesicles (Marion et al., 1987; Marion et al., 1989).

Although the aggregated state of the ligand in multilayered liposomes and the diversity of the molecular species present in the lipid fractions gives rise to a complex situation, it was possible to get good fittings of the fluorescence titration experiments with simple binding models. It is first interesting to note that interactions between puroindoline and phospholipid vesicles is a noncooperative phenomenom with tight binding (K_d in the 10^{-7} to 10^{-8} M range) whereas cooperativity with lower binding properties (K_d in the 10^{-3} to 10^{-5} M range) characterizes the interaction of puroindolines with glycolipids layers. Cooperativity and similar K_d values are also observed with long chain lysoPC (Wilde et al., 1993; Husband et al., 1995). The major difference between wheat phospholipids and both glycolipids and lysoPC is that wheat phospholipids are negatively charged because they are composed of >60% NAPE. Because puroindolines are positively charged, these results suggest that electrostatic interactions contribute to the tight and noncooperative binding of puroindolines to phospholipids layers. Fluorescence

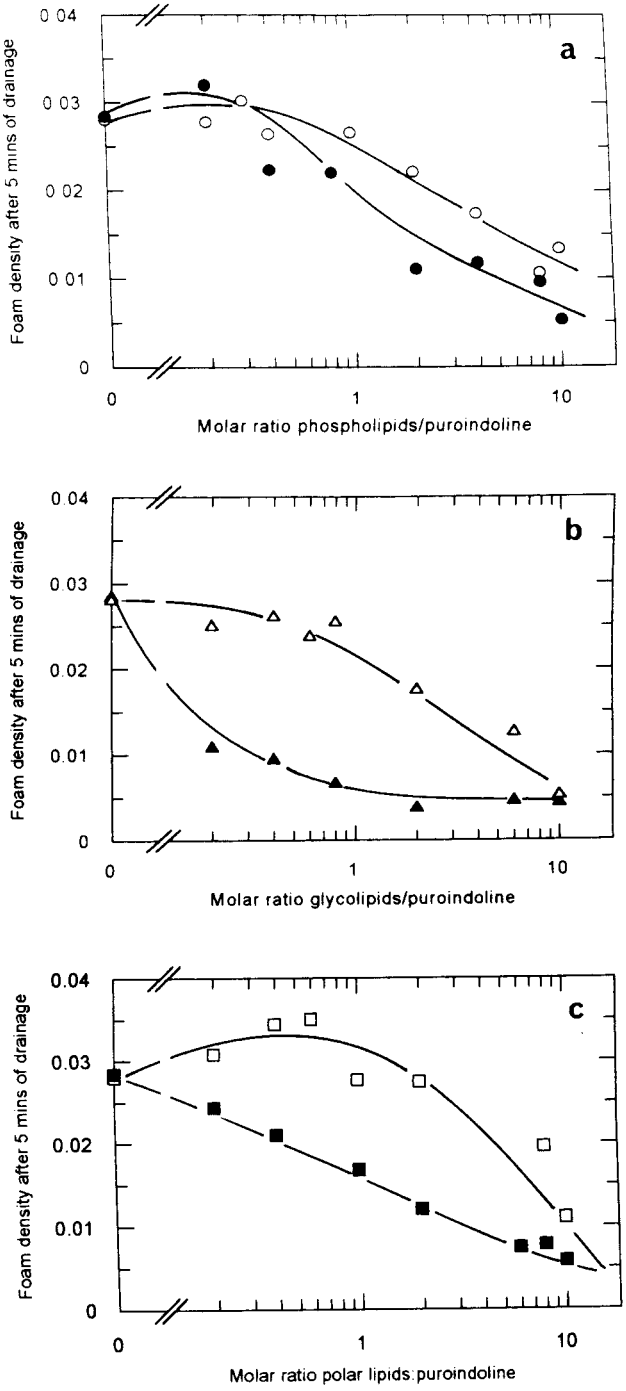

Figure 4. Foam density of PIN-a and PIN-b (0.3 mg mL^{-1}) after 5 min of drainage as a function of lipids:puroindoline molar ratios of 0 to 10 in 10 mM sodium phosphate buffer at pH 7.0. (a) PIN-a (o) and PIN-b (●) with wheat phospholipids; (b) PIN-a (△) and PIN-b (▲) with wheat glycolipids, (c) PIN-a (□) and PIN-b (■) with total wheat polar lipids

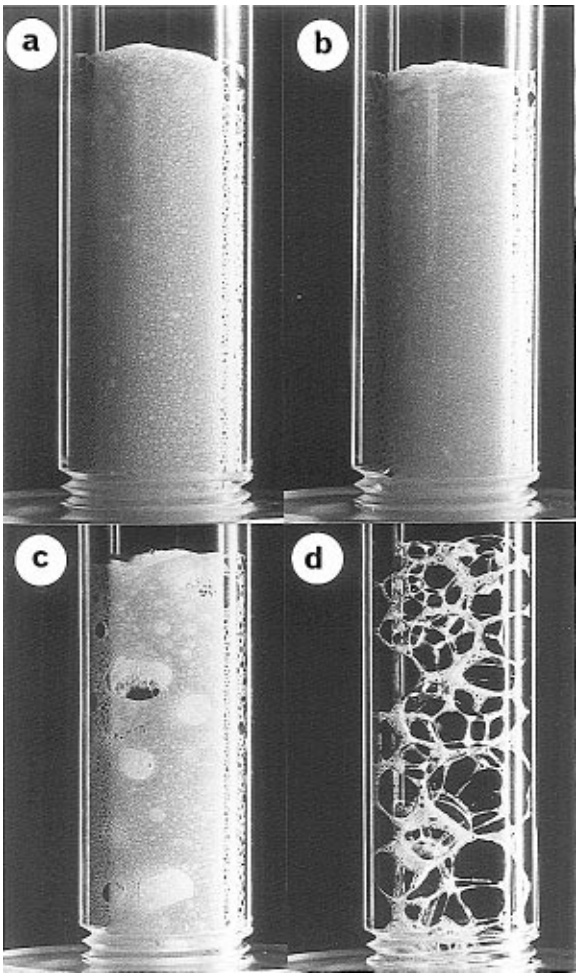

Figure 5. Puroindoline foams at the end of bubbling. (a) PIN-a and (b) PIN-b alone (0.3 mg mL^{-1}); and (c) PIN-a and (d) PIN-b with glycolipids ($R_i = 6$).

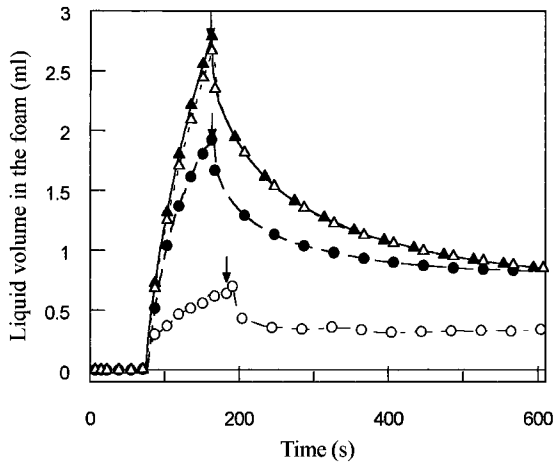

Figure 6. Evolution of liquid volume in the foam versus time for PIN-a (0.3 mg mL^{-1}, ▲) and egg white proteins in phosphate buffer (1.25 mg.mL^{-1}, ●); in presence of wheat total polar lipids at $R_i = 1$ with PIN-a (△) and egg white proteins (○). The R_i was determined in ovalbumin equivalent), and the arrows indicate end of bubbling.

polarization of DPH shows that puroindolines can penetrate more deeply in phospholipid bilayers than in glycolipid bilayers. This result is in agreement with the fact that puroindoline binding to phospholipids leads to the highest variations in tryptophan fluorescence intensity. Therefore, in the case of negatively charged lipids, formation of electrostatic bonds between protein and lipid head group strengthens hydrophobic interactions between protein and lipids. On the contrary, the low increase of both DPH polarization and fluorescence intensity observed for glycolipid−puroindoline interactions suggest that PIN-a and PIN-b do not penetrate deeply in the glycolipid bilayer. Glycolipids contain hexoses that could interact between them and impede protein−lipid interaction through steric effects. Therefore, weak hydrophobic bonds are probably formed between puroindolines and glycolipids. 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−nonpolar interface

of lipid bilayers (Landolt-Marticorena et al., 1993; Schiffer et al., 1992; Hu et al., 1995). The truncated tryptophan-rich domain in PIN-b is probably responsible for the lower affinity of PIN-b than PIN-a for glycolipids. The highest affinity of PIN-a for glycolipids and neutral lysoPC could, therefore, be mainly due to the formation of hydrogen bonds between glycolipid hydroxyls or carbonyls and indole NH.

The interaction of PIN-a with phospholipids and total wheat polar lipids, a mixture of ~25% phospholipids and 75% glycolipids, leads to strictly identical K_d and n values, suggesting that (1) dispersion of the glycolipid−phospholipid mixture in water gives rise to mixed liposomes and not to an heterogeneous population of phospholipid and glycolipid liposomes and (2) PIN-a interacts both with glycolipids and phospholipids in a mixed liposome. Furthermore the n values ($n = 4$) are close to those found for lysoPC ($n = 5$; Wilde et al., 1993) showing that the interaction occurs with all the lipid molecules of multilayered liposomes. Concerning wheat phospholipids, it is important to keep in mind that they are also a mixture of negatively charged and neutral phospholipids (mainly PC and PE). Therefore, the identity of binding sites for lysoPC and total wheat phospholipids and polar lipids suggests that PIN-a interacts with all lipids in mixed liposomes composed of neutral and negatively charged lipids. In the case of PIN-b, the loose interaction with neutral lipids (K_d in the 10^{-3} M range) is apparently inconsistent with the high affinity (K_d in the 10^{-8} M range) for total wheat phospholipids. This inconsistency could suggest that PIN-b interacts preferentially with negatively charged lipids. Such a selectivity is generally observed with basic membrane active proteins (Dufourcq et al., 1982) and is probably responsible for the apparent higher number of binding sites and a lower effect on DPH polarization in PIN-b than in PIN-a.

The tight binding of puroindolines to polar lipids and especially to negatively charged phospholipids should induce a preferential partitioning of these proteins in dough polar lipids. This preferential partitioning explains why, as for membrane proteins, nonionic detergents are necessary to extract puroindolines although these proteins are quite water soluble after purification (Blochet et al., 1991). This tight binding supports the hypothesis that the affinity of puroindolines for the surface of starch granules is mediated by residual starch surface lipids (Greenblatt et al., 1994) and is in agreement with the fact that most of these proteins are not bound to starch (Jolly et al., 1993).

As previously suggested (Marion and Clark, 1996), the most important functional properties of puroindolines should be related to their ability to stabililize, along with polar lipids, the air−water interfaces in doughs . In this study, egg white proteins were chosen as a standard of a protein ingredient used for its good foaming properties on one hand and as a non lipid binding protein in the other hand. In regard to egg proteins, the stability of puroindoline foams are considerably higher especially in the presence of wheat polar lipids. The destabilizing effect of wheat polar lipids on egg white protein foams is due to a competitive adsorption phenomenom that takes place between wheat polar lipids and egg white proteins (Clark et al., 1991; Sarker et al., 1995). Lipid binding obviously impairs this competitive effect because (1) it is impossible to get 35 mL of foam with egg white proteins at $R_i = 5$ although it still possible to have a foam at $R_i = 10$ with both puroindolines, and (2) PIN-a is more efficient than PIN-b at preventing foam destabilization by wheat polar lipids.

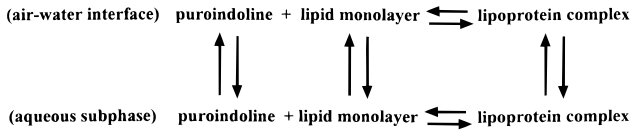

Figure 7. Equilibrium distribution of puroindolines and wheat polar lipids between aqueous subphase and air−water interface.

As for the lipid binding studies just described, the foaming behavior of puroindoline−wheat polar lipids is highly complex. It is important to keep in mind that the adsorption of closed bilayers and spreading in an open monolayer at the air−water interface is energetically unfavorable compared with the micellar lysoPC previously used (Wilde et al., 1993; Husband et al., 1995). This energetic unfavorability is due to the strong lipid−lipid interactions at the bilayer surface, and spreading can occur if some defects in lipid packing are created in the bilayers. In fact, an equilibrium is created between lipid bilayer in the subphase and the lipid monolayer at the air−water interface and such an equilibrium is governed by the lipid−lipid interactions in both bilayers and monolayers (see Marion and Clark, 1996, for a review). In the case of wheat polar lipids, stable foam was formed in the concentration range investigated in this study, suggesting that the equilibrium is largely shifted towards the formation of stable bilayer liposomes into the subphase. With puroindolines, a complex multiequilibrium phenomenon takes place that governs the partitioning of free and bound puroindolines between the aqueous subphase and the air−water interface and that is governed by the stability of lipid−puroindoline foams (Figure 7). Foam stability can be due both to tight protein−protein interactions and to the formation of strong lipid−protein films as previously shown for PIN-a alone and with lysoPC (Wilde et al., 1993). The latter mechanism is not so important with wheat polar lipids as with lysoPC because only slight or no synergistic effect is observed with wheat polar lipids. However, it is not negligible and can account for the resistance to foam destabilization that is closely related to the strength of lipid−protein interactions. Thus, in the case of PIN-a, foam destabilization does not start before $R_i = 1-2$ with phospholipids and total polar lipids, whereas a slight but significant destabilization occurs with glycolipids below $R_i = 1$. For PIN-b, efficiency of lipids to induce foam destabilization and K_d are of the same order, with glycolipids > total polar lipids > phospholipids. Foam destabilization can arise from three different phenomena: (1) competitive adsorption between wheat polar lipids and puroindolines; (2) an increase of lipid spreading at air−water interface by puroindolines that intensifies competitive adsorption and/or leads to the formation of a lipoprotein film less stable than the protein film; and (3) trapping of puroindolines by liposomes present in the aqueous subphase. For PIN-b, competitive adsorption should dominate because foam stability decreases dramatically at low R_i although almost all protein is free. For PIN-a, the mechanism of protein trapping by liposomes into the subphase should dominate because at R_i values of >1, foam stability decreased linearly as the content of free PIN-a decreases. It is noteworthy that from fluorescence titration data at $R_i = 1$, ~85% of PIN-a is still free in solution whereas at $R_i = 5$, 80−90% of PIN-a is bound to lipids.

The results obtained in this work show that puroindolines and especially PIN-a bound wheat polar lipids more tightly than the monoacyl lipids previously studied (Wilde et al., 1993; Husband et al., 1995). The foam stability of the mixed lipoprotein systems is quite sastisfactory at lipid concentrations well above concentrations that impair foaming properties of nonlipid binding proteins. These properties of puroindolines suggest that they should play a major role in the formation and retention of the gas phase in doughs by preventing lipid destabilization of protein foams formed on mixing by the soluble nonlipid binding proteins present in the aqueous phase of dough.

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