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Protein–lipid interactions in gluten elucidated using acetic-acid fractionation

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ABSTRACT

Protein–lipid interactions in dough have an important impact on the quality of bakery products. Understanding of protein–lipid interactions in gluten can enhance the development of technological solutions to improve the breadmaking quality of flour as well as the functional properties of gluten. In this study, acetic acid at two different concentrations was used for treating and fractionating gluten. The impact of these procedures on the distribution of lipid components was measured. Acetic acid was able to dissociate non-polar lipids from the gluten protein matrix. Upon fractionation monomeric proteins (predominantly gliadins) and phospholipids were high in the 0.01 M acetic acid soluble fraction. The subsequent fractionation step using 0.1 M acetic acid resulted in an increased amount of high-molecular-weight glutenin subunits (HMW-GS) in the soluble fraction, along with more non-polar lipids and glycolipids in both the free and bound lipid extracts. The distribution of lipid classes demonstrates that non-polar lipids are either associated with the glutenin polymeric network through hydrophobic interactions or entrapped within the gluten matrix. The results also indicate that in gluten, glycolipids are likely to be associated with glutenins through both hydrophobic interactions and hydrogen bonds whilst phospholipids preferentially interact with gliadins and lipid binding proteins.

1. Introduction

Protein–lipid interactions in wheat flour dough systems are known to be important because both the lipid and the protein, primarily the gluten protein, govern the breadmaking quality of flour (MacRitchie, 1983; MacRitchie & Gras, 1973; McCormack, Panozzo, Bekes, & MacRitchie, 1991). Flour lipids comprise as little as 2% of flour weight but they do have a positive effect on dough formation and loaf volume in breadmaking (MacRitchie, 1983), on the volume and softness of steamed bread (Pomeranz, Huang, & Rubenthaler, 1991) and on the texture of short-dough biscuits (Papantoniou, Hammond, Scriven, Gordon, & Schofield, 2004). In a series of definitive experiments (MacRitchie, 1983) polar lipids were found to have a positive effect on the loaf volume whilst non-polar lipids were detrimental. The non-starch lipids are known to contribute to the loaf volume of bread, especially of cultivars unsuited for breadmaking (McCormack et al., 1991), whilst the free fatty acid component of non-polar lipids can cause the depression of loaf volume (MacRitchie, 1983). Glycolipids in the polar lipid extract are most effective in improving loaf volume, whilst phospholipids only increase volume in the presence of added shortening (MacRitchie, 1983).

The interactions of protein and lipid can be probed through the changes in the levels and distribution of free and bound lipids in flour, dough and gluten. The definitions of free and bound lipids are based on the solvent extractability of lipid. Free lipids are the lipids that can be extracted with non-polar solvents including petroleum ether, hexane or diethyl ether, whilst bound lipids can only be extracted with polar solvents such as ethanol or a mixture of an alcohol and water. More than half of the free lipids in flour become associated with the gluten protein during dough mixing (Chung, 1986). The association could involve either physical entrapment or binding of lipids to protein (Carr, Daniels, & Frazier, 1992; Chung, 1986; Marion, Roux, Akoka, Tellier, & Gallant, 1987). As a result, some of the flour lipids can no longer be extracted with the non-polar solvents used for free lipid extraction.

Studies on the presence of specific chemical and/or physical interactions between gluten protein and lipid have reported that the protein and lipid might be associated through chemical interactions involving polar and hydrophobic bonding. A model of lipid binding in dough has been proposed by Pomeranz and Chung (1978), suggesting that dough is strengthened by the ability of polar lipids to be associated with protein and starch. In contrast, other studies using physical techniques, particularly phosphorus nuclear magnetic resonance spectroscopy and freeze-fracture electron

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microscopy, concluded that lipid is retained within the gluten in a relatively non-specific way and that the interaction of protein and lipid in gluten may involve the physical entrapment of lipid and also polar or ionic bonding between the components (Carr et al., 1992; Marion et al., 1987). Because the extracted lipids in gluten protein fractions can depend on the fractionation method used, the distribution of lipids between alcohol- or detergent-based fractions may reflect the solubility of lipids in the solvent rather than native protein–lipid interactions (Carr et al., 1992). Thus, in studying protein–lipid interactions the fractionation of the gluten protein with non-alcohol and non-detergent agents is preferable.

For many decades, it has been known that although, gluten is a water-insoluble substance, it can be largely dissolved in a dilute acetic acid solution. This solubilisation could be due to the effect of the acidic conditions on changing structure and net charge of the protein molecules (Damodaran, 1996) as well as the disruption of hydrogen bonds within the gluten matrix (Wrigley, Bekes, & Bushuk, 2006). Various acetic acid concentrations have been used to selectively separate gliadin and glutenin fractions (Berot, Gautier, Nicolas, Godon, & Popineau, 1994). Treating gluten with these varying acid concentrations could affect the gluten structure, thereby impacting on the distribution of free and bound lipids, thus reflecting the interaction of protein and lipid in gluten.

Therefore, in this study, a sequential fractionation method was developed with two concentrations of acetic acid (0.01 and 0.1 M) to selectively separate gluten protein with different ratios of monomeric-to-polymeric components, whilst simultaneously fractionating protein/lipid aggregates into more clearly defined groups. Lipid classes in the free and bound categories were investigated for the gluten fractions, together with the protein profiles of the acetic acid treated gluten and gluten fractions. The results provided further understanding of the association of monomeric and polymeric proteins with specific lipid classes in the gluten matrix.

2. Experimental

2.1. Materials

Wheat flour used for this study was produced from the Australian cultivar Lang, harvested in 2004, kindly milled and provided by Allied Mills (Kensington, VIC, Australia). The flour contained 14% moisture, 12.8% protein (as is basis) and 1.54% lipids (as is basis). Gluten was prepared from flour on a laboratory scale. Flour (300 g) was mixed with water (180 ml) for 1 min at setting 1 using a Hobart mixer N-50G (Hobart Corporation, USA), and then at setting 2 for 2.5 min to form a dough. This was rested in water for 30 min and hand washed with water (3×5 l) to remove starch. Wet gluten was collected, freeze-dried and ground using a coffee grinder. All studies were carried out using a gluten control sample (50 g) which was prepared by removing free lipids using petroleum ether (4×200 ml). The resultant gluten was placed in a fume cupboard for 3 days to allow evaporation of solvent residue and then stored at −20 °C.

2.2. Preparation of acetic acid treated gluten and gluten fractions

Acetic acid treated gluten samples were prepared by mixing the gluten control with 0.01 or 0.1 M acetic acid at a ratio of 1:20 (w/v) and 20 °C using an Ultra-Turrax T25 mixer (Janke and Kunkel GmbH & Co., Staufen, Germany) at a speed of 9500 rpm for 2 min. The treated gluten was then freeze-dried, ground to powder and stored at −20 °C.

Gluten protein fractions were obtained using sequential acetic acid treatment. The gluten control was first treated at 20 °C with 0.01 M acetic acid using the same conditions as above, then centrifuged at 24,000*g* at 4 °C for 15 min. The supernatant, termed the "0.01 M acetic acid soluble fraction" was removed by decanting and the insoluble material was re-extracted with 0.1 M acetic acid using the same conditions and centrifuged. The second supernatant, termed the "0.1 M acetic acid soluble fraction", was removed from the remaining pellet. The 0.01 M acetic acid soluble, 0.1 M acetic acid soluble and insoluble fractions were freeze-dried separately, then ground to powder and stored at −20 °C. The fractions were prepared in triplicate from the same gluten control material.

The protein contents of the gluten control, the acetic acid treated glutens and gluten fractions were determined according to the AACC standard method 46-30 (AACC, 1995) using a Leco system (FP2000, Leco Corporation, USA).

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

The protein composition of the gluten samples was analysed using SDS–PAGE based on the method of (Kasarda, Woodard, & Adalsteins, 1998) with some modifications. SDS–PAGE was performed using a NuPAGE gradient precast gel (4–12% gradient) bis–tris (10×10 cm^2) in a Novex Xcell Mini cell (Invitrogen, Victoria, Australia). MES buffer containing 50 mM MES, 50 mM tris base, 0.1% SDS, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.3, was used as the electrophoresis running buffer. The protein sample (10 mg) was dissolved in the sample buffer (1 ml) containing 11.25 mM tris–HCl, pH 8.5, 3.6% SDS, 50 mM dithiothreitol, 18% glycerol, and 0.0025% bromophenol blue and heated at 85 °C for 10 min. For the analysis of proteins in non-reducing conditions, dithiothreitol was omitted from the sample buffer. The protein samples (30 μg of protein per well) and the protein standard markers (Novex Mark 12, Invitrogen, Victoria, Australia) were loaded onto the gel. Electrophoresis was performed at 100 V for 60 min, followed by 120 V for 50 min, for the proteins under reduced conditions, and at 80 V for 75 min, followed by 90–95 V for 75 min, for proteins under non-reduced conditions. Proteins were visualised by staining with Coomassie Brilliant Blue R-250 solution (0.1% in 40% methanol and 10% acetic acid) for 25 min then de-stained overnight with a solution of 10% methanol and 7.5% acetic acid.

2.4. Size exclusion high-performance liquid chromatography (SE-HPLC)

The ratios of monomeric and polymeric proteins in gluten control, acetic acid treated glutens and the gluten fractions were determined using SE-HPLC (Batey, Gupta, & MacRitchie, 1991). The sample (10 mg) was extracted at 20 °C with 0.05 M phosphate buffer, pH 6.9 containing 0.5% SDS (1 ml) for 60 min using a combination of vortexing, plus sonication (Bransonic 221, Branson, Shelton, USA) at 50/60 Hz for 10 min with heating at 50 °C. The protein extract was centrifuged at 15,000*g* for 5 min and filtered through a 0.45 μm filter (Bonnet, NSW, Australia). The protein extract (20 μl) was injected into the Biosep-SEC S4000 column (Phenomenex, NSW, Australia), connected to a Shimadzu HPLC system (Shimadzu, Japan) consisting of LC-10A model pumps, SIL-20A automatic sampler and a model SPD-20A UV–visible detector. Samples were eluted under isocratic conditions using a solvent containing 0.1% trifluoroacetic acid in 50% acetonitrile at a flow rate of 0.5 ml/min, monitored by UV detection at 214 nm.

2.5. Lipid extraction

Lipids in the gluten control, acetic acid treated gluten and gluten fractions were extracted using petroleum ether and ethanol at a ratio of 1:20 (sample/solvent, w/v). The extraction consisted of two steps: petroleum ether at 20 °C, twice; followed by ethanol at 70 °C also twice; to obtain free and bound lipids, respectively. Solvents were removed from the lipid extracts using a rotary

evaporator at 40 °C for petroleum ether and at 75 °C for ethanol, followed by further drying under nitrogen. Lipid extracts were dissolved to a concentration of 10 mg/ml with chloroform for the free lipids, or with chloroform/methanol (1:1 v/v) for the bound lipids, and then stored at −20 °C.

2.6. Lipid class analysis by HPLC

The composition of lipid extracts was determined using HPLC. The lipid extract (5–20 μl) was injected onto PVA Sil column (5 μm, 250 × 4.6 mm; YMC, Japan), connected with an HP 1050 series HPLC (Hewlett Packard, Japan) equipped with a PL-ELS 1000 Evaporative Light Scattering Detector (Polymer Laboratories, UK). This was operated at 40 °C for nebulisation and at 80 °C for evaporation with a gas flow of 1.0 ml/min. The HPLC eluent system consisted of eluent A (2,2,4-trimethyl pentane:isopropanol (98:2 v/v) containing 0.2% of *N*-ethyl morpholine:glacial acetic acid (2:0.45 v/v), eluent B (dichloromethane), eluent C (methanol containing 0.2% of *N*-ethyl morpholine:glacial acetic acid (2:0.9 v/v)) and eluent D (methanol). Lipid classes were separated at an eluent flow rate of 1 ml/min with a gradient starting with 40%/60% A/B for 3 min, following by 10%/60%/30% A/B/C for 15 min, 40%/60% B/C for 10 min, 20%/80% B/D for 3 min, and ending with 100% eluent B for 2 min. The standard curves of individual lipid standards derived using 5–100 mg of the lipids were linear with R^2-values in the range of 0.93–0.99. The lipid classes were calculated as the proportion of the total lipid extract.

2.7. Statistical analyses

Comparisons of lipid content, proportions of lipid classes and protein content within the acetic acid gluten fractions were performed using ANOVA, at a single factor in Microsoft Excel.

3. Results

3.1. Acetic acid concentration and the distribution of proteins and lipids

In order to investigate the impact of acetic acid on the distribution of lipids and protein, samples of gluten control were separately mixed with 0.01 and 0.1 M acetic acid and then freeze-dried. There were no differences in the protein profiles between the gluten control and the acetic acid treated glutens on reduced and non-reduced SDS–PAGE gels (results not shown). Similarly, SE-HPLC showed no change in the ratio of monomeric-to-polymeric proteins as a result of the acetic acid treatment (Table 1). Protein recoveries were 92.3% and 93.7% for the glutens treated with 0.01 and 0.1 M, respectively (Table 1). These results were expected because acetic acid is a weak acid and it can alter the folding protein structure, thereby affecting the protein solubility (Damodaran, 1996). Under the conditions of the current treatment, acetic acid cannot cause the hydrolysis of peptide bonds, hence, the protein pattern of acetic acid treated gluten was expected to be similar to that of the gluten control.

Table 1
Protein and lipids in gluten control and acetic-acid treated gluten.[a]

	Gluten control	0.01 M acetic-acid treated gluten	0.1 M acetic-acid treated gluten
pH	6.27	4.72	3.42
Protein content (%, w/w)	70.6	75.2	74.2
Protein recovery (%, w/w)		92.3	93.7
Ratio monomeric-to-polymeric proteins by SE-HPLC	1.80	1.78	1.76
Free lipids (%, w/w)	0.23	1.11	1.25
Non-polar lipids	0.23	1.11	1.17
Glycolipids	0.00	0.00	0.08
Phospholipids	0.00	0.00	0.00
Bound lipids (%, w/w)	5.47	3.10	3.31
Non-polar lipids	2.76	1.20	1.11
Glycolipids	2.09	1.64	1.84
Phospholipids	0.62	0.26	0.36
Total lipids (%, w/w)	5.70	4.20	4.60

[a] Total lipids are calculated based on the total of free lipids and bound lipids; in calculating the lipid classes the components were: non-polar lipids including triacylglyceride (TAG), diacylglyceride (DAG) and monoacylglyceride (MAG); glycolipids including monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG); phospholipids including phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC).

The lipids in the gluten control were predominantly present as bound lipids (96% of total lipids) because most of free lipids had been removed by the preliminary extraction with petroleum ether. After treating gluten with acetic acid, the amount of free lipids in the acetic acid treated gluten (1.11–1.25%) was higher than that in the gluten control (0.23%) (Table 1). Treatment with acetic acid released much of the bound lipids as free lipids, increasing the proportion of free lipids from only 4% (gluten control) to about 27% in both treated gluten samples. This important change indicates that acetic acid can dissociate some lipids (particularly non-polar and glycolipids) from the gluten matrix, possibly by changing the surface charge of the protein (Damodaran, 1996) or the interaction of monomeric and polymeric proteins in the gluten polymer network.

The changes in the proportions of free and bound lipids after acetic acid treatment are primarily due to a redistribution of non-polar lipids; nearly half of the non-polar lipids of the gluten control became free lipids (Table 1). Only 4% of the glycolipids became free lipids after treatment at the high acetic acid concentration (0.1 M), indicating that the higher acid condition has greater effectiveness in dissociating the more polar lipids from the gluten matrix. Nevertheless, no phospholipids were rendered free after acetic acid treatment, although some bound phospholipids were lost as a result of the treatment.

3.2. Sequential fractionation with acetic acid

Having established the effects of acetic acid treatment on lipid composition, the respective acid concentrations were used for sequential fractionation of the control gluten to effect the partition of gluten proteins amongst the extracts obtained with 0.01 M acetic acid, 0.1 M acetic acid and the final residue. The gluten was distributed amongst the three fractions by thirds, approximately. Whilst only 48% (w/w) protein was found in the insoluble fraction, both the 0.01 and 0.1 M acetic acid soluble fractions contained high amounts of protein, 90.2% (w/w) and 83.2% (w/w), respectively. In addition, the relative amounts of the total gluten protein solubilised into 0.01 M acetic acid and subsequently into 0.1 M acetic acid were similar, 34.3% and 39.3%, respectively. This was expected as the residual insoluble, non-protein matter (largely starch and fibre) in gluten would remain as insoluble material throughout the fractionation process.

The results from SE-HPLC (Fig. 1, I) showed that the fractionation procedure was successful in separating monomeric from polymeric proteins, with the ratio falling from 2.4 (0.01 M acetic acid extract) to 1.05 in the residue. Similar results had been reported by MacRitchie, Kasarda, and Kuzmicky (1991) when fractionating wheat flour using diluted hydrochloric acid. The separation of monomeric and polymeric proteins in the current fractionation procedure was also evident from SDS–PAGE patterns for reduced samples (Fig. 1), which show that the dominant protein bands had apparent molecular weights (MWs) between 30 and 50 kDa in the 0.01 M acetic acid soluble fraction (Fig. 1, II, lane

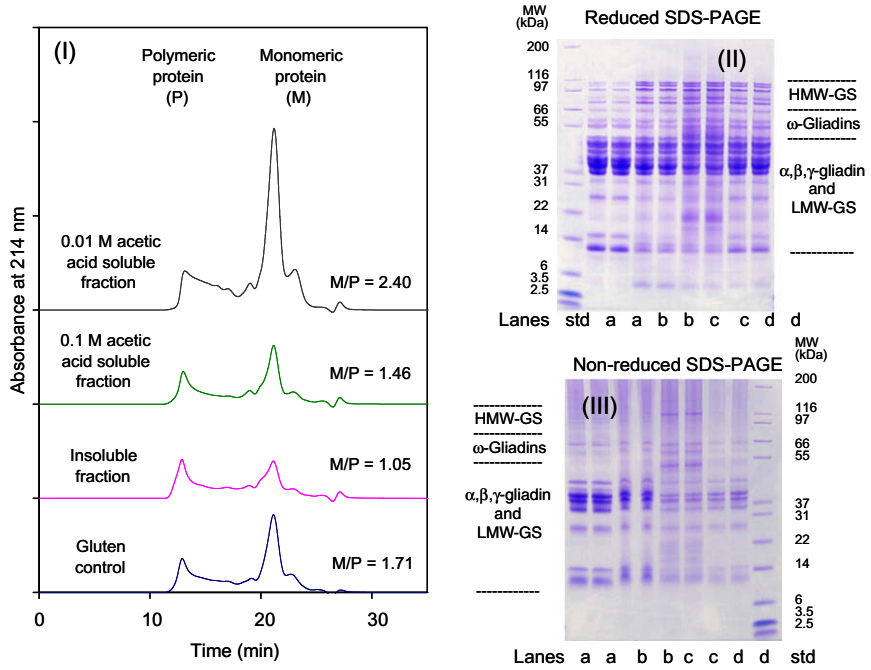

Fig. 1. Characterisation of proteins in 0.01 M acetic-acid soluble fraction (a), 0.1 M acetic-acid soluble fraction (b), insoluble fraction (c) and gluten control (d) using SE-HPLC (I) and SDS–PAGE at reduced (II) and non-reduced conditions (III).

a), whilst there were more protein bands above 66 kDa in the 0.1 M acetic acid soluble fraction (Fig. 1, II, lane b). Both SE-HPLC (Fig. 1, I) and non-reduced SDS–PAGE (Fig. 1, III) showed that the residual fraction had a high proportion of polymeric glutenin proteins. The near absence of protein bands in the region of 30–55 kDa on the unreduced gel of the insoluble fraction (Fig. 1, III, lane c) indicates that this fraction contained little monomeric gliadin. The appearance of the protein bands in this region under reduced conditions (Fig. 1, II, lane c) was due to the presence of low-molecular-weight glutenin subunits (LMW-GS) in this fraction.

3.3. Distribution of lipids in sequential acetic acid fractions

The overall recovery of lipids after gluten fractionation was approximately 100% (Table 2). The distribution of lipids between the three fractions was significantly different. Only a small amount of lipids (0.6 g in 100 g gluten) were found in the 0.01 M acetic acid fraction, accounting for 12% of the total lipids in the gluten control. It was noted that the lipid content of this fraction was also lower than the amount of lipids in the original flour (1.5%, w/w flour). More than half of the lipids from the gluten control (59% or 2.9 g of lipid in 100 g defatted gluten) was found in the 0.1 M acetic acid soluble fraction, four times more than the amount of lipids in the 0.01 M acetic acid soluble fraction (Table 2). Approximately 29% of lipids from gluten control (1.4 g of lipids in 100 g defatted gluten) remained in the insoluble fraction. Taking into account the protein content in the three fractions, the lipid contents as a function of total protein in the each fraction were equivalent to 1.5, 7.5 and 6.1 g lipids per 100 g protein.

The total amount of free lipids increased greatly as a result of acetic acid fractionation (Table 2), accounting for 27% of the total lipids; this increase was similar to that observed for acetic acid treatment (Table 1). Consequently, the amount of bound lipids was significantly lower in the gluten fractions than in the gluten control (Table 2). Within the three acetic acid fractions (Table 2), the amount of free lipids was lowest in the 0.01 M acetic acid soluble and residue fractions (1% and 6% of total lipids, respectively). Most of the free lipids (21%) were found in the 0.1 M acetic acid soluble fraction. In contrast, for the previous experiment when gluten was separately treated with 0.01 and 0.1 M acetic acid, the amount of free lipids was found to increase to approximately, 25% of the total lipids at both acetic acid concentrations (Table 1). It is likely that free lipids dissociated from the protein in the first fractionation with 0.01 M acetic acid had remained in the pellet. Following the second fractionation with higher acetic acid concentration (0.1 M), the free lipids were primarily recovered in the

Table 2
Distribution of protein and lipids in sequential acetic-acid fractions.[A]

	Gluten control	Sequential acetic-acid fractionation			
		0.01 M acetic-acid soluble fraction[B]	0.1 M acetic-acid soluble fraction[B]	Insoluble fraction[B]	Total
Fractionation yield (% w/w)	–	27.3 ± 0.1[a]	33.7 ± 0.6[b]	34.3 ± 0.2[b]	95.3 ± 0.6
Protein content (% w/w fraction)	71.6 ± 0.2	90.2 ± 0.4[a]	83.2 ± 0.8[a]	48.0 ± 0.4[b]	–
Protein recovery (% w/w gluten)	–	34.3 ± 0.1[a]	39.3 ± 0.7[b]	23.0 ± 0.2[c]	96.6 ± 0.6
Free lipids (% w/w gluten)	0.19 ± 0.05	0.06 ± 0.01[a]	1.00 ± 0.02[b]	0.29 ± 0.13[c]	1.34 ± 0.13
Bound lipids (% w/w gluten)	4.55 ± 0.16	0.55 ± 0.05[a]	1.88 ± 0.09[b]	1.13 ± 0.07[c]	3.56 ± 0.11
Total lipids (% w/w gluten)	4.74 ± 0.06	0.61 ± 0.04[a]	2.87 ± 0.11[b]	1.42 ± 0.09[c]	4.90 ± 0.02

[A] Results are the means of triplicate analyses.
[B] Superscript letters are used to show statistically significant differences between means within the same row ($p < 0.01$): values followed by the same letter are not significantly different.

Table 3
The distribution of lipid classes as free and bound lipids following sequential fractionation.[A]

	Sequential acetic-acid fractionation		
	0.01 M acetic-acid soluble fraction[B]	0.1 M acetic-acid soluble fraction[B]	Insoluble fraction[B]
Free lipids			
Non-polar lipids	57 ± 10[a]	607 ± 33[b]	293 ± 131[a]
TAG	(86)	(64)	(85)
DAG	(10)	(19)	(9)
MAG	(3)	(17)	(6)
Glycolipids	0[a]	311 ± 19[b]	0[a]
MGDG	0	(73)	0
DGDG	0	(27)	0
Phospholipids (PE, PS + PC)	0[a]	76 ± 3[b]	0[a]
Bound lipids			
Non-polar lipids	82 ± 11[a]	629 ± 57[b]	415 ± 26[c]
TAG	(59)	(68)	(73)
DAG	(5)	(7)	(7)
MAG	(36)	(25)	(20)
Glycolipids	103 ± 5[a]	956 ± 43[b]	530 ± 31[c]
MGDG	(68)	(76)	(76)
DGDG	(33)	(24)	(24)
Phospholipids	367 ± 55[a]	291 ± 2[a]	182 ± 22[b]
PE	(0.3)	(1)	(4)
PI	(0.7)	(3)	(3)
PS + PC	(12)	(84)	(84)
LPE	(34)	(0)	(0)
LPC	(53)	(11)	(9)

[A] Values are expressed in units of milligram of lipids in 100 g of gluten. TAG, triacylglyceride; DAG, diacylglyceride; MAG, monoacylglyceride; MGDG, monogalactosyl diglyceride; DGDG, digalactosyl diglyceride; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine.

[B] Data in parentheses are the proportion expressed as a percentage for the lipid class. Superscript letters are used to show statistically significant differences between means within the same row ($p < 0.01$): values followed by the same letter are not significantly different.

0.1 M acetic acid soluble fraction. The fraction also contained a high proportion of glutenin (Table 2).

The total non-polar lipids (triacylglycerides (TAG), monoacylglycerides (MAG) and diacylglycerides (DAG)) were the major components of free lipids isolated from all fractions, being highest in the 0.1 M acetic acid soluble fraction (Table 3). A significant amount of glycolipids (monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG)), together with small amounts of phospholipids (phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylcholine (PC)) were also found as part of the free lipids in the 0.1 M acetic acid fraction (Fig. 2a–c and Table 3). This was even more evident when the amount of free lipids was expressed as a percentage of protein in the fraction (Fig. 3).

The lipid class composition of the bound lipids showed that the 0.01 M acetic acid fraction contained a relatively high proportion of phospholipids, whilst the 0.1 M acetic acid soluble and insoluble fractions contained relative high amounts of glycolipids (Fig. 2d–f and Table 3). Similar lipid distribution patterns were observed for the bound lipids in the 0.1 M acetic acid fraction and the insoluble fraction (Fig. 2e and f). By considering the amount of protein in these two fractions, the differences in their lipid distributions were greatly reduced (Fig. 3).

4. Discussion

In the presence of water during the mixing process, flour lipids become bound to or associated with gluten protein through hydrophobic and polar forces (Chung, 1986; Frazier, 1983; MacRitchie, 1983). Following the separation of starch from gluten protein, most of the lipids are not extractable by non-polar solvents. Treating gluten with acid, even under mild conditions, causes changes in the surface charge of the protein, thereby changing the hydrophobicity and structural conformation of the protein. It is also possible that under acidic conditions, the polymeric network of gluten protein is disrupted as a result of weakening of the hydrogen bonds between gliadin and glutenin proteins (Wrigley et al., 2006). Evidence of this is seen in the observations on the proteins solubilised at the lower concentration of acetic acid (0.01 M). These were rich in gliadins (monomeric proteins) (Fig. 1, II, lane a) whilst protein remaining (extracted at or remaining after the higher acetic acid concentration) consisted largely of glutenins (polymeric proteins) (Fig. 1, II, lanes b and c). It has previously been suggested that the separation of monomeric and polymeric proteins primarily depends on the concentration of acetic acid as well as the ratio of gluten and solvent (Berot et al., 1994). In the gluten matrix, α-, β-, γ- and ω-gliadins are not able to form inter-chain linkages through disulphide bonds. Hence their structure and contribution to the gluten matrix is stabilised by hydrophobic interactions and hydrogen bonds. In contrast, disulphide bonds, hydrogen bonds and hydrophobic interactions contribute directly to the formation of inter- and intra-chain bonds between HMW-GS, LMW-GS and other proteins (Bushuk, 1998; Shewry, Halford, & Tatham, 1992; Shewry & Tatham, 1997). This would explain why monomeric proteins could be dissociated from the gluten matrix at the lower acetic acid concentration (0.01 M) whilst polymeric proteins required a much higher acetic acid concentration (0.1 M) in order to increase their solubility or extractability.

The effect of acetic acid on the structure of gluten protein, in turn, resulted in increased amounts of lipids, particularly the non-polar lipids being extracted by a non-polar solvent. After monomeric proteins were dissociated from the gluten matrix at the lower concentration of acetic acid (0.01 M), free lipids components, primarily non-polar lipids preferentially remained in the glutenin-rich fraction (pellet) rather than in the gliadin-rich fraction (0.01 M acetic acid soluble fraction). This is consistent with non-polar lipids being entrapped between gliadin and glutenin in the gluten network (Marion et al., 1987). The non-polar lipids, being insoluble in the aqueous solution of a very dilute acetic acid concentration, might remain at the surface of the glutenins through the hydrophobic interactions after gliadins were removed. Consequently, these lipids could be extracted with non-polar solvents including petroleum ether. It was also observed that non-polar lipids were distributed evenly between free and bound lipids in each protein fraction (Table 3), indicating that non-polar lipids were associated with the gluten protein in a non-specific way. The results were in agreement with the findings by Marion et al. (1987) who suggested that all gluten lipids existed in lipid vesicles entrapped within the gluten network. However, this study demonstrated that the entrapment of lipids in gluten occured only for the non-polar lipids, whilst polar lipids appeared to interact with gluten protein through specific binding mechanisms.

The dissociation of polymeric proteins from the gluten matrix coincided with the appearance of free glycolipids. This suggests that glycolipids might be entrapped within the polymeric proteins. However, the distribution of free and bound glycolipids in the sequential acetic acid fractions could be interpreted in another way. Free glycolipids were only found in the 0.1 M acetic acid soluble fraction (Table 3). Of the total glycolipids, a large amount of glycolipids occurred as bound lipids in the 0.1 M acetic acid soluble and the insoluble fractions whilst only a small amount of glycolipids was present in the free lipids extract from the 0.1 M acetic acid soluble fraction. Glycolipids can interact with protein through hydrophobic interactions as well as via hydrogen bonds (Hoseney, Finney, & Pomeranz, 1970). As acetic acid is able to disrupt hydrogen bonds or to change the surface charge of protein, the hydrogen

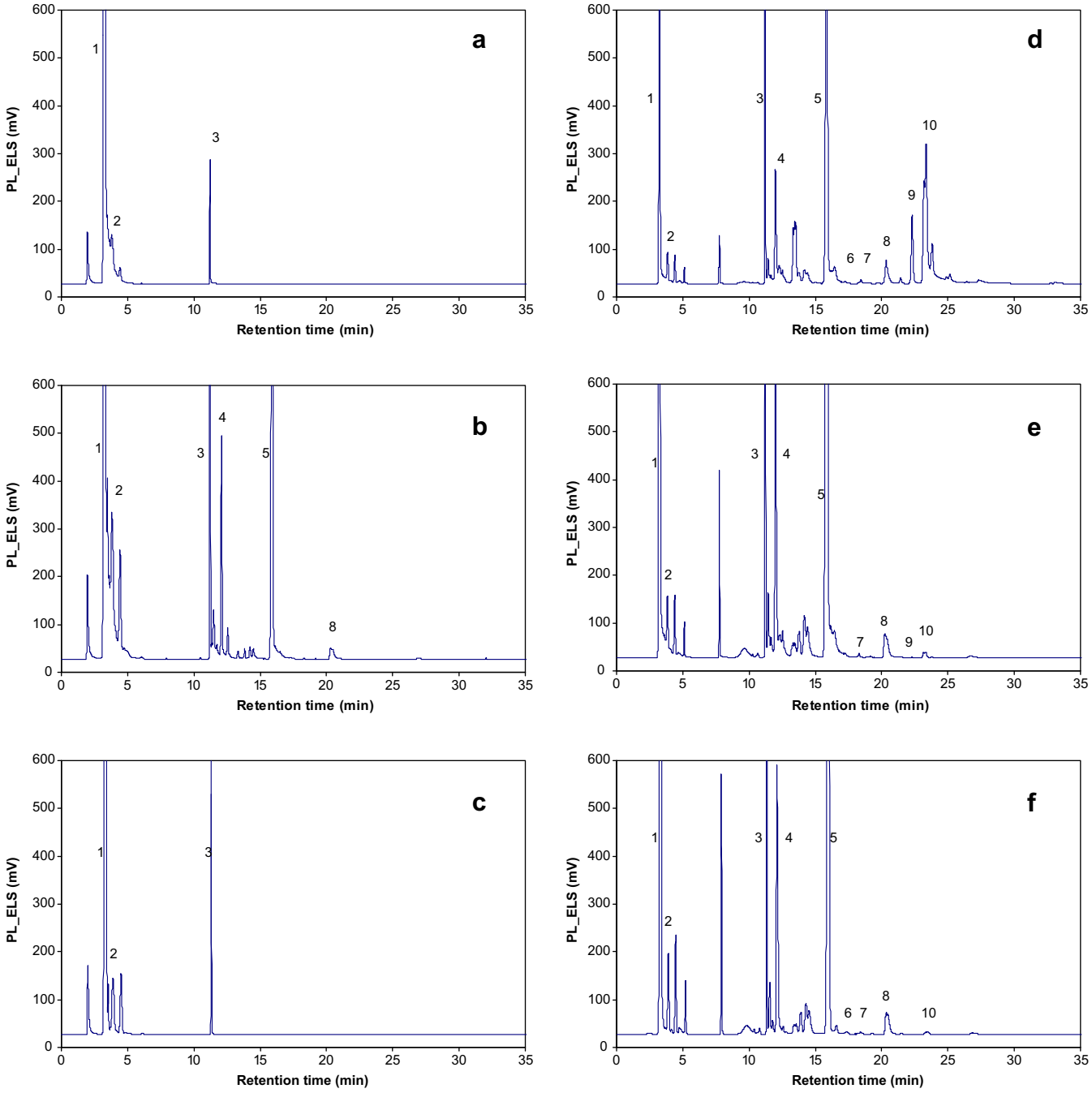

Fig. 2. HPLC chromatogram of free (at left) and bound (at right) lipid extracts of 0.01 M acetic acid soluble fraction (a, d), 0.1 M acetic acid soluble fraction (b, e) and insoluble fraction (c, f). The lipid composition was identified using a series of lipid standards consisting of TAG (1), DAG (2), MAG (3), MGDG (4), DGDG (5), PE (6), PI (7), PS and PC (8), LPE (9) and LPC (10).

bonds between glycolipids and glutenins might be weakened, therefore the glycolipids would became extractable in petroleum ether. This explanation was further supported by the absence of glycolipids in the free lipid extract of the acetic acid insoluble fraction, the protein of which showed less effect of acetic acid on the surface charge and hydrogen bonds. The evidence supports the occurrence of interactions between glycolipids and glutenins, rather than the entrapment of the lipids within the structure of the gluten matrix.

The relatively high level of bound lipids, found in the 0.1 M acetic acid soluble fraction indicates that most of the lipids were preferentially associated with the gluten protein solubilised at the high acetic acid concentration. The interactions of protein and lipids in the 0.1 M acetic acid soluble fraction and the insoluble fraction were likely to be similar as they had the same pattern of protein and lipid distribution (Table 3). In addition, there was no significant difference in the amount of bound lipids per gram of protein although the protein content in these fractions was different (Table 3). As much of the protein in the insoluble fraction was polymeric glutenin (Fig. 1), the association of protein with bound lipids in the 0.1 M acetic acid soluble and the insoluble fractions primarily involved HMW-GS and LMW-GS.

Non-polar lipids and glycolipids were the major bound lipids in the 0.1 M acetic acid soluble and the insoluble fractions containing

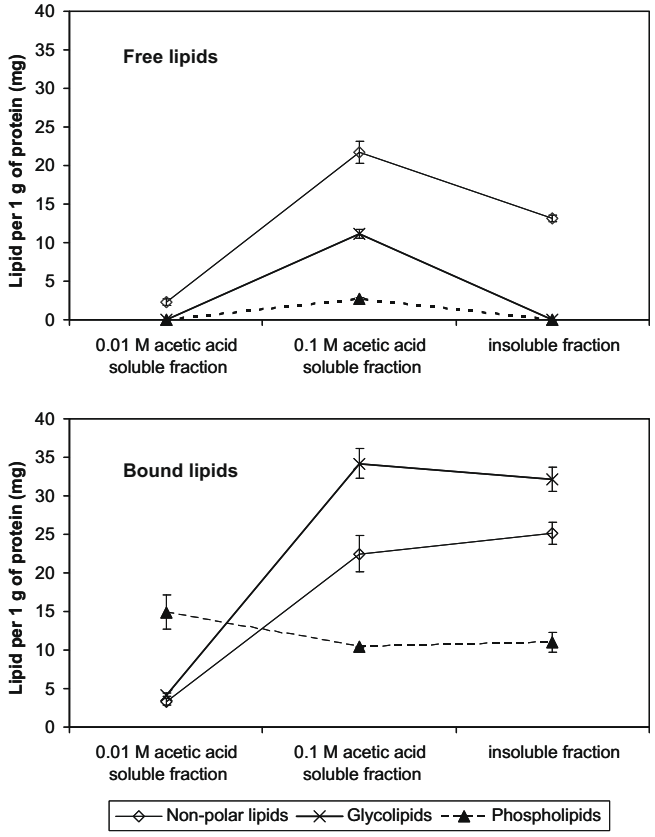

Fig. 3. Relationship of lipids and protein in acetic-acid gluten fractions from sequential extraction.

a high proportion of glutenins. This suggests these lipids were likely to be associated with glutenins. However, non-polar lipids may not specifically interact with glutenins, but rather they may be locked within the gluten matrix formed between gliadins and glutenins. Glycolipids interacted with glutenins which was in the 0.01 M acetic acid soluble as well as in the insoluble fraction as demonstrated by the presence of similar amount of bound glycolipids per gram of protein (Fig. 3). In addition, the similar ratio between MGDG and DGDG was found in both 0.1 M acetic acid soluble and the insoluble fractions (Table 3). Furthermore, the ratio of MGDG and DGDG in the free lipid extract of the 0.1 M acetic acid soluble fraction is the same as in the bound lipid extract. The results indicate that acetic acid at 0.1 M concentration has an effect on polymeric glutenins, weakening the association of glutenins and glycolipids, so that some of the glycolipids can be extracted with petroleum ether.

Fig. 3 shows that a high proportion of bound phospholipids were found in the 0.01 M acetic acid soluble fraction. Since this fraction contained a high amount of gliadins, it is reasonable to suggest that phospholipids are preferentially associated with gliadins. Furthermore, it was observed that the level of phospholipids decreased from the 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble and further to the insoluble fraction (Fig. 3), whilst the densities of protein bands at MW of approximately, 12, 16, 26.8 and 42 kDa also decreased (Fig. 1, top right, lanes a–c). These results suggest that these proteins might preferentially associate with phospholipids. It is likely that the 0.01 M acetic acid soluble fraction contained a number of proteins being able to associate with phospholipids. This appears to confirm various observations of previous studies. Proteins with similar MW values have been found in the S protein fraction known to be able to associate with polar lipids (Zawistowska, Bietz, & Bushuk, 1986). Puroindolines are proteins with a MW of 12.8 kDa and they have been found to have a high affinity for phospholipids and glycolipids (Dubreil, Compoint, & Marion, 1997). Chloroform–methanol soluble proteins having a MW of 12–13 kDa are known to be lipoproteins which accumulate with lipids extracts (Carr et al., 1992).

The distribution of lipid classes as free and bound lipids in the gluten fractions was more specifically related to the types of proteins in the fraction, rather than to the protein quantity. The evidence from this study indicates that flour lipids, particularly non-polar lipids were retained within the gluten network through hydrophobic forces, involving the physical entrapment of lipids within the protein network. Glycolipids were found to be associated with glutenins through both hydrophobic interactions and hydrogen bonds. There was no evidence to suggest that glycolipids were associated with gliadin proteins. On the other hand, the distribution of phospholipids in the gluten fractions indicates that they probably interacted with either gliadins or lipid binding proteins within the gluten matrix.

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