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Effects of flour chlorination on soft wheat gliadins analyzed by reversed-phase high-performance liquid chromatography, differential scanning calorimetry and fluorescence spectroscopy

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Chlorinated soft wheat flours produce better quality cakes. In this study, changes in surface hydrophobicity of gliadins due to flour chlorination were analyzed. Results obtained from RP-HPLC of gliadins showed intervarietal differences, but intravarietal differences in the gliadin patterns of chlorinated and unchlorinated flours were difficult to discern because of the large number of peaks eluted. However, three-dimensional plots of retention time, wavelength and absorbency showed differences among gliadin patterns of unchlorinated and chlorinated flours within a variety, suggesting conformational changes and consequently alteration in hydrophobicity of gliadins due to chlorination. The DSC analyses of gliadins extracted from unchlorinated and chlorinated flours showed higher denaturation peak transition temperatures (T_d) and lower enthalpies of transition (ΔH) in some varieties, indicating hydrophobicity and conformational changes. Fluorescence spectroscopy measurements with an extrinsic probe, 1-anilino-8-naphthalene sulfonic acid (ANS), showed increases in hydrophobicity of gliadins from chlorinated flours, confirming conformational changes in gliadins due to chlorination.

INTRODUCTION

Reactions of chlorine gas (Cl_2) with wheat flour induce three basic changes: bleaching of pigments, primarily carotenoids, reduction in pH, and chemical modification of flour components (Tsen & Kulp, 1971). Work in our laboratory (Yamamoto *et al.*, 1994) and others (see Hoseney *et al.*, 1988; Gough *et al.*, 1978) have shown that high-ratio cakes made from chlorinated flour have finer textures, better contours and higher volumes than cakes from unchlorinated flour. In addition, studies on effects of Cl_2 on the starch component of flour have shown that chlorinated starch, which undergoes oxidative depolymerization (Huang *et al.*, 1982; Johnson *et al.*, 1980), imbibes more water, increases in viscosity (Kulp & Tsen, 1972), and binds oil (Seguchi, 1984). It has been suggested that the increased water absorbency is due to increased permeability of the starch granule surface (Conforti & Johnson, 1992).

Among the cake flour fractions, chlorinated gluten was more effective than chlorinated prime starch in raising quality scores of cakes baked (Sollars, 1958). Although the amount of starch granule protein was shown to increase with chlorination (Seguchi, 1990), the total quantity of protein in the chlorinated flour did not differ significantly from that of unchlorinated flour (Conforti *et al.*, 1993). Conforti *et al.* (1993) also reported decreases in linoleic and linolenic acid contents due to chlorination and suggested that the tenderness of angel food cakes made from chlorinated flour may be due to the combined effects of proteins and fatty acids.

Tsen and Kulp (1971), based on an increase in extractability of proteins in water and acetic acid, inferred that the inter- and intramolecular bonds of the protein molecules are broken by the action of Cl_2, causing increased dispersibility of proteins. These authors also reported that Cl_2 caused degradation of the aromatic amino acids and oxidation of sulfhydryl groups.

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Gaines *et al.* (1988) showed that chlorination imparted cohesiveness to soft flour dough. Among the gluten proteins, gliadins impart cohesiveness. They are more hydrophobic than glutenins (Pomeranz, 1988) and thus can contribute to protein aggregation and to binding of lipids and glycolipids. Therefore, an understanding of the effect of chlorine on gliadins at the molecular level may explain in part chlorinated flour's ability to hold a high ratio of shortening. In this study, we investigated the effects of chlorination on qualitative changes in gliadins by differential scanning calorimetry (DSC), which can monitor enthalpy changes due to protein unfolding and phase transition (Mateo, 1982; Arntfield *et al.*, 1981). We also used reversed-phase high-performance liquid chromatography (RP-HPLC) and fluorescence spectroscopy to investigate changes in surface hydrophobicity of gliadins due to flour chlorination.

MATERIALS AND METHODS

Chemicals and reagents

Protein sequencing grade anhydrous trifluoroacetic acid was obtained from Sigma Chemical Company (St Louis, MO, USA), HPLC grade acetonitrile was obtained from Fisher Scientific Company (St Louis, MO, USA), 1-anilino-8-naphthalene sulfonic acid magnesium salt (ANS) was obtained from Eastman Kodak Company (Rochester, NY, USA). All other chemicals were reagent grade and obtained from Sigma Chemical Company.

Flours

Approximately 3.0 kg of grain of each of soft wheat varieties, Caldwell (soft red, OH, USA), Dynasty (soft red, OH, USA), Frankenmuth (soft white, MI, USA) and Lewjain (soft white, WA, USA), tempered to 14% moisture, were milled by a Miag-Multomat mill into 45% patent flours. Resulting flour protein contents were 6.8, 7.9, 6.6 and 8.1%, respectively, on a 14% moisture basis. The flour samples were chlorinated to pH 4.8 and pH 4.3 according to Kissell and Marshall (1972). A portion of each flour sample was set aside as an unchlorinated control.

Osborne protein fractionations

Gliadins were extracted from 10 g of each flour sample according to the modified sequential Osborne procedure of Chen and Bushuk (1970). Gliadin fractions were freeze-dried and used for DSC and fluorescence spectroscopy analyses.

Extraction of gliadins for RP-HPLC

Two hundred milligrams of flour were dispersed in 800 μl extraction solvent containing HPLC grade 70% v/v ethanol, 5% v/v isopropyl alcohol and 5% v/v methanol. The flour–solvent mixture was held for 1 h at room temperature with occasional shaking on a vortex shaker and then centrifuged at 10000*g* for 15 min in a Sorvall MC 12C (Du Pont Instruments, Wilmington, DE, USA) bench top centrifuge maintained at room temperature. The supernatant obtained was filtered through a 0.45 μm filter (Millipore, MA, USA) and used for HPLC injection.

RP-HPLC of gliadins

Chromatography of gliadins was performed on a Millennium 2010 HPLC work-station consisting of a Waters 600E multisolvent delivery system (Waters, Milford, MA, USA), a temperature control module and a 996 photodiode array detector. A Zorbax 300 SB-C8 (MACMOD Analytical, Inc., PA, USA) reversed-phase column (250×4.6 mm i.d.) connected to a guard column (125×4.0 mm i.d.) with the same packing material was used to separate gliadins. The solvents used were A: 10% HPLC grade acetonitrile with 0.1% trifluoroacetic acid (TFA) and B: 90% HPLC grade acetonitrile with 0.1% TFA. The solvents were degassed and purged with helium at the rate of 8 ml/min during the run. The gradient program used for gliadin separation, monitored from 190 to 340 nm, is listed in Table 1. The injection volume was 20 μl. The solvent flow rate was 1.0 ml/min and the column was maintained at 60°C with a Waters temperature control module. Each sample was analyzed in duplicate. Peak area integration was done with the Millennium 2010 software program.

Differential scanning calorimetry (DSC) of the gliadin fraction

A Du Pont 2920 DSC unit (Wilmington, DE, USA) was used to measure denaturation peak transition (T_d) temperatures and enthalpy changes (ΔH) of gliadins extracted from unchlorinated and chlorinated flours. Approximately 6–7 mg of samples, as is moisture basis, were weighed and sealed in an aluminium sample pan (TA instruments, Newcastle, DE, USA) by an encapsulating press. All samples were analyzed in duplicates.

Table 1. Solvent gradient for reversed-phase high-performance liquid chromatography

Time (min)	Solvent B[a] (%)	Solvent gradient
0	18	Initial conditions
0–4.0	18–24	Linear
4.0–50.0	24–46	Linear
50.0–54.0	46–47	Linear
54.0–58.0	47	Isocratic
58.0–63.0	47–18	Linear
63.0–68.0	18	Equilibrate to initial conditions

[a]Ninety percent acetonitrile + 0.1% trifluoroactic acid.

Samples were heated from 25 to 225°C and scanned at 10°C/min; an empty sample pan was used as a reference. The DSC cell was flushed with nitrogen at 50 ml/min to maintain an inert environment during the measurements. The T_d and ΔH were determined by the General V4.1C software program which controlled the DSC unit.

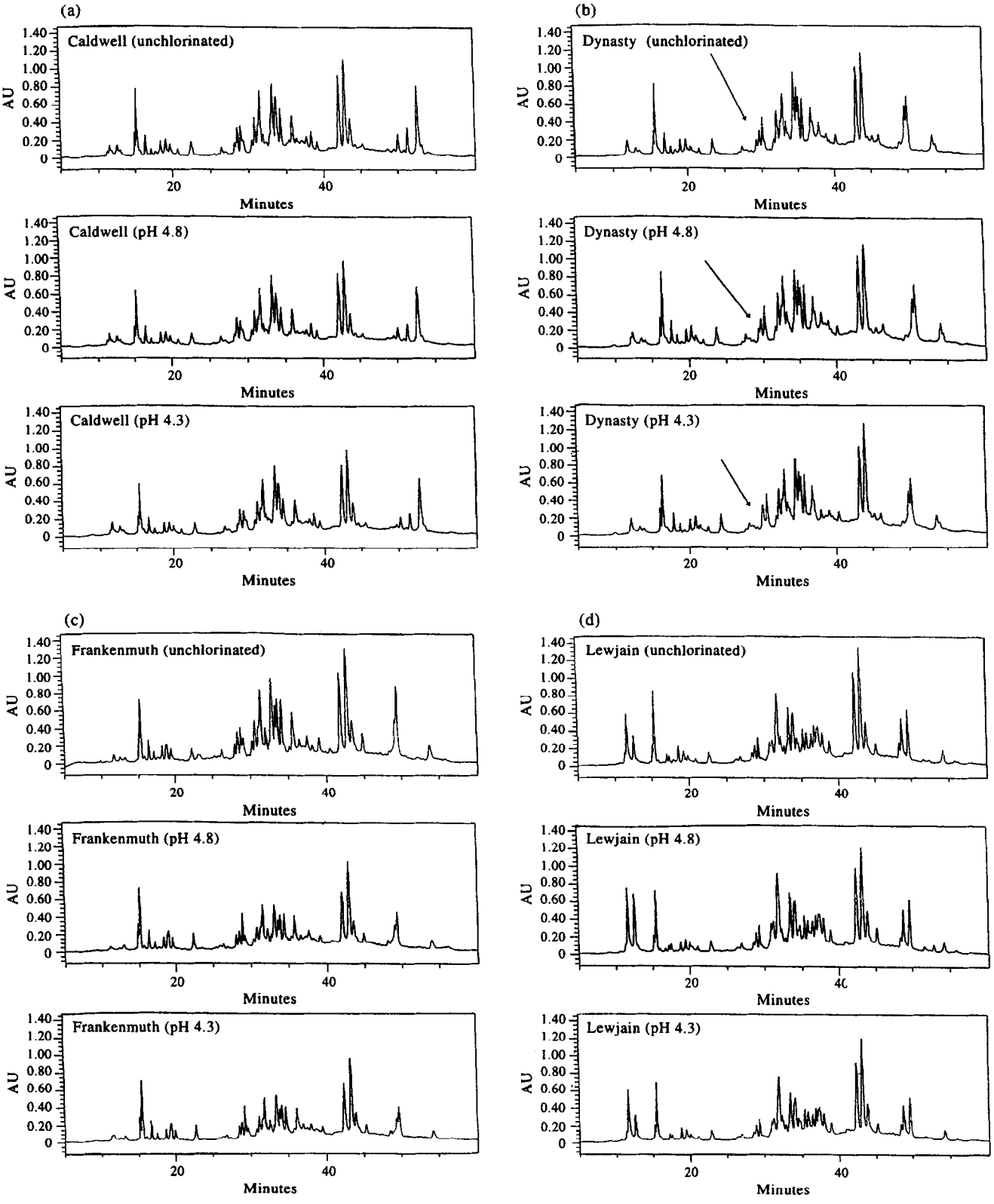

Fig. 1. Reversed-phase high-performance liquid chromatography of gliadins extracted from unchlorinated and chlorinated flours from (a) Caldwell, (b) Dynasty, (c) Frankenmuth and (d) Lewjain varieties.

Hydrophobicity changes in gliadins determined by fluorescence spectroscopy

Hydrophobicities of gliadins extracted from unchlorinated and chlorinated flours were determined using an extrinsic fluorescence probe 1-anilino-8-naphthalene sulfonic acid (ANS) according to Lakkis and Villota (1992). To establish a standard plot for estimating hydrophobicities, five concentrations (0.1 mg/ml to 1 mg/ml in distilled water) of gliadins (from flour chlorinated to pH 4.8) were prepared by mixing on a vortex shaker and holding for 2 h at room temperature. Ten microliters of ANS (8 mM in 0.1 M phosphate buffer) were then added to the 2 ml gliadin dispersion and fluorescence intensity was measured with a SLM 4800 spectrophotometer (Urbana Champaign, IL, USA) connected to a data acquisition and operating system from On-Line Instrument Systems (Bogart, GA, USA). Fluorescence measurements were made in duplicate using semi-micro quartz fluorescence cuvettes (4×10 mm) held in a thermostable block maintained at 22°C. The excitation wave length was 390 nm (excitation wave length of ANS) and emission was scanned between 400–600 nm. Slit width was 4 nm for both excitation and emission. The fluorescence intensity based on emission maximum at 479 nm was measured. The slope of the plot of fluorescence intensity vs. gliadin concentration was used to calculate the hydrophobicity index (Lakkis & Villota, 1992) of gliadins. The concentration of gliadin dispersions from unchlorinated and chlorinated samples was 1 mg/ml in distilled water and fluorescence intensities were measured as above in duplicate.

Statistical analysis

The RP-HPLC chromatographic peak area data were analyzed with two-factor (treatment×variety) ANOVA using the SuperANOVA Software program (Abacus Concepts, Inc., Berkeley, CA, USA). Mean comparisons were done according to least standard deviation (LSD) at the 5% level of significance.

RESULTS AND DISCUSSION

RP-HPLC of gliadins

Gliadins extracted from unchlorinated and chlorinated flours were analyzed by RP-HPLC to examine the effects of chlorination on this group of storage proteins. Figures 1(a) to 1(d) show the RP-HPLC patterns (at 210 nm) of gliadins extracted from unchlorinated and chlorinated (pH 4.8 and pH 4.3) flours of the four wheat varieties. Previous studies (Bietz, 1983; Weiser *et al.*, 1987) have reported separation of gliadins based on their hydrophobicities. The elution order of gliadins is: ω-gliadins, α-gliadins and γ-gliadins. In the present study, intervarietal differences in gliadins, indicative of genotype differences, are apparent from comparison of the peak patterns of unchlorinated samples. Within a variety, the differences in the peak patterns among unchlorinated and chlorinated flour samples were difficult to discern because of the large number of peaks eluted. However, on careful examination small differences in gliadins of unchlorinated and chlorinated flours were evident. For example, in the case of Dynasty [Fig. 1(b)] there was loss of a peak eluting at about 30 min (arrows) in the flour sample chlorinated to pH 4.3.

In order to determine whether there were any quantitative differences among peak areas of gliadins from unchlorinated and chlorinated samples, the gliadin peaks were segmented over three retention time periods, 5–25 min, 25–40 min and 40–55 min, since the majority of peaks eluted appeared to be grouped into these three regions representing ω-gliadins, α-gliadins and γ-gliadins, respectively. It was hypothesized that conformational changes and/or breakdown products due to the action of chlorine on these gliadins at the molecular level would show up in their peak areas (Table 2).

Within each variety, the peak area of each of the chlorinated samples eluted during 5–25 min was slightly higher than that of the respective unchlorinated sample. However, only the variety Lewjain showed a significant difference ($p=0.026$) between unchlorinated and chlorinated gliadins for peaks eluted during 5–25 min.

Table 2. Relative mean areas of gliadin peaks in unchlorinated and chlorinated flours*

Variety	Peak area† (%)								
	5–25 min‡			25–40 min‡			40–60 min‡		
	A[d]	B[d]	C[d]	A[d]	B[d]	C[d]	A[d]	B[d]	C[d]
Caldwell	15.36[a]	16.18[a]	16.40[a]	51.20[b]	49.00[b]	49.16[b]	33.44[c]	34.81[c]	34.73[c]
Dynasty	11.66[a]	12.94[a]	12.67[a]	48.08[b]	53.55[b]	53.96[b]	40.26[c]	33.54[c]	33.34[c]
Frankenmuth	13.30[a]	16.28[a]	15.02[a]	52.53[b]	51.04[b]	50.85[b]	33.56[c]	32.67[c]	34.45[c]
Lewjain	13.14[a]	15.61[b]	14.73[b]	48.72[a]	46.42[ab]	46.59[b]	38.13[a]	37.63[a]	38.68[a]

*Percent of total area of all peaks eluted during the three time segments.
†Average of two HPLC runs. Means in the horizontal rows for each variety within each time segment having same letters (as superscripts) were not significantly different at the 5% level.
‡Elution time in three segments.
[d]Flour sample: A = unchlorinated, B = chlorinated to pH 4.8, C = chlorinated to pH 4.3.

The differences in peak areas of unchlorinated and chlorinated gliadins eluted during 25–40 min and 40–55 min did not show a uniform increase or decrease in all varieties. For Lewjain, Frankenmuth and Caldwell, the peak areas of gliadins eluted during 25–40 min for chlorinated samples were lower than those of the unchlorinated, and the variety Lewjain showed a significant difference between peak areas of gliadins from unchlorinated flours and from flours chlorinated to pH 4.3 ($p = 0.037$).

Rozing (1993) used three-dimensional plots of retention time, wavelength and absorbency to show minor peaks and shoulders for studying identity and homogeneity of proteins. In the present study, three-dimensional plots were obtained through a photo diode array detector. Through these three-dimensional plots, the subtle changes in gliadin patterns of unchlorinated and chlorinated flours were evident. As examples, three-dimensional patterns of gliadins extracted from unchlorinated and chlorinated flours for variety Lewjain are shown in Fig. 2(a) and (b). The three-dimensional plots demonstrated a distinct difference among gliadins from chlorinated and unchlorinated flours in this variety and also in Caldwell, Frankenmuth and Dynasty (figures not shown). Seguchi (1985) showed that gelatin, bovine serum albumin, or ovalbumin formed a hydrophobic water-insoluble thin film after chlorination. In the present study, the modifications in peak patterns of chlorinated samples may indicate conformational changes and hence changes in their surface hydrophobicities.

The DSC of gliadins extracted from unchlorinated and chlorinated flours

In the present study, the denaturation transition temperatures (T_d) of gliadins ranged from about 121–140°C (Table 3, Fig. 3). Myers (1990) reported a T_d range of 60–80°C for a group of proteins. The author also suggested that high T_d values are expected for proteins with a high proportion of hydrophobic residues involved in the denaturation mechanism. Therefore, the high T_d values obtained in this study may be due to high hydrophobicities of gliadins. Harwalkar and Ma (1987) reported that, at pH values far from the isoelectric point, the protein is partially unfolded due to disruption of hydrogen bonds and hydrophobic interactions. In the present study, the T_d and ΔH values of chlorinated and unchlorinated samples were not significantly different ($p > 0.05$). However, some chlorinated gliadins had slightly higher T_d values (except variety Frankenmuth) than unchlorinated gliadins, indicating an increase in hydrophobicities of those chlorinated samples.

Arntfield *et al.* (1990) suggested that enthalpy (ΔH) values related to conformational changes of proteins. The ΔH values of gliadins from flours chlorinated to pH 4.3 were lower than those from unchlorinated flours (except for variety Frankenmuth). It is likely that chlorination caused partial unfolding of gliadins so that less heat energy was required to dissociate or disrupt the ordered structure of gliadins extracted from chlorinated samples as evidenced by their lower ΔH values. These decreases in ΔH values of chlorinated samples suggest conformational changes due to chlorination.

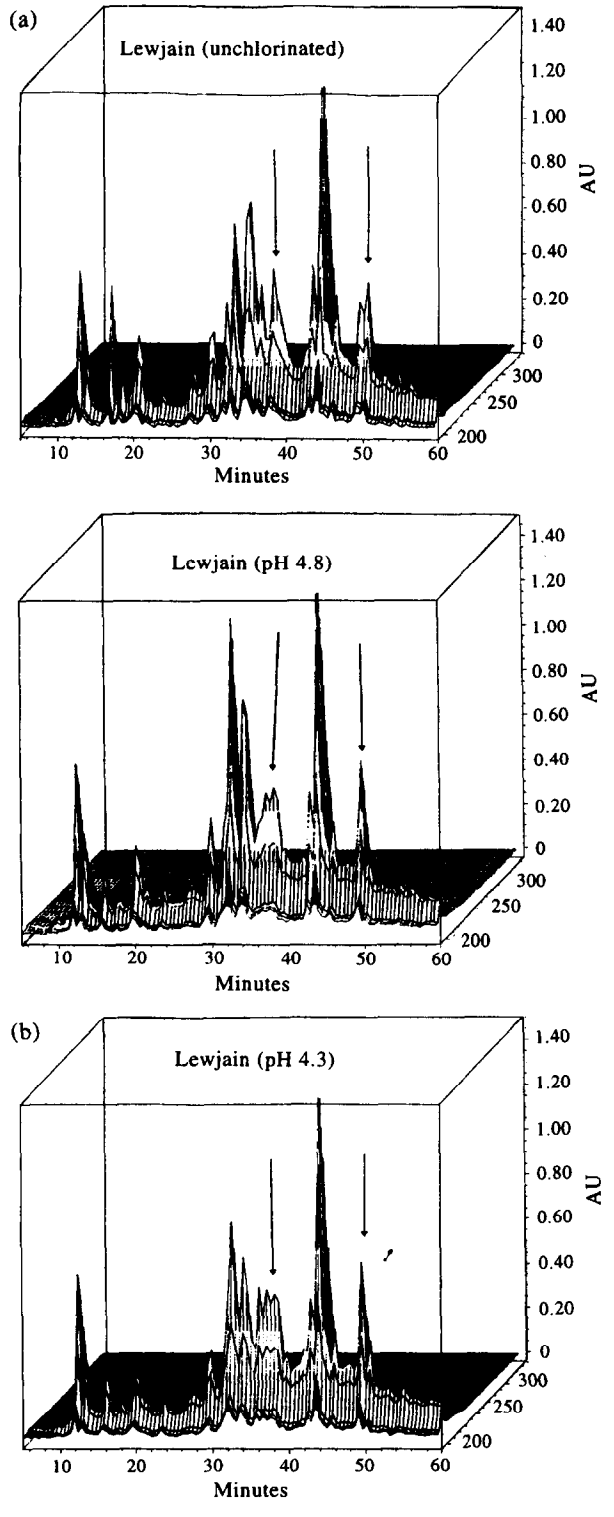

Fig. 2. A three-dimensional plot of RP-HPLC of gliadins from unchlorinated and chlorinated flours from variety Lewjain: (a) unchlorinated (top) and chlorinated (middle) to pH 4.8 and (b) (bottom) chlorinated to pH 4.3.

Changes in hydrophobicity of gliadins determined by fluorescence spectroscopy

The fluorescence emission maxima for gliadins with the extrinsic probe, 1-anilino-8-naphthalene sulfonic acid (ANS) was 479 nm. Without this probe, little emission was observed; ANS has been reported to display fluorescence when bound to macromolecules or when dissolved in non-polar solvents (Lakowicz, 1983). The surface hydrophobicity indices of gliadins measured by the ANS probe increased progressively with the degree of chlorination (Fig. 4). This is indicative of a change in the conformational structure of gliadins due to flour chlorination and a subsequent exposure of hydrophobic sites to the ANS probe. In comparison to RP-HPLC and DSC, fluorescence spectroscopy provided a more sensitive measurement of changes in hydrophobicities of gliadins due to chlorination.

In summary, the results of RP-HPLC of gliadins showed small differences among peak areas of gliadins from unchlorinated and chlorinated flours. The differences among the three-dimensional patterns of RP-HPLC analyses of gliadins from unchlorinated and chlorinated flours were more pronounced, suggesting changes in structural conformation of gliadins due to chlorination. The lower enthalpy of transition (ΔH) values of chlorinated gliadins indicated partial unfolding due to chlorination, and their higher T_d values suggested increased hydrophobicities. The increase in hydrophobicity of chlorinated gliadins resulting from conformational changes was confirmed by fluorescence spectroscopy. This information should be useful for further understanding flour chlorination and how the process alters proteins to subsequently affect cake baking quality.

Table 3. Differential scanning calorimetry data of unchlorinated and chlorinated gliadin fractions from four soft wheat varieties[a]

Variety	T_d[b]	ΔH[c]
Caldwell		
Unchlorinated	125.7	200.9
pH 4.8	129.9	190.2
pH 4.3	121.5	196.5
Dynasty		
Unchlorinated	129.9	188.5
pH 4.8	129.3	220.9
pH 4.3	140.2	186.7
Frankenmuth		
Unchlorinated	129.8	173.3
pH 4.8	124.2	185.0
pH 4.3	128.4	196.6
Lewjain		
Unchlorinated	134.7	192.0
pH 4.8	135.3	194.5
pH 4.3	137.2	169.5

[a]Mean of two DSC measurements.
[b]Denaturation transition peak temperature in °C.
[c]Enthalpy of transition in J/g.

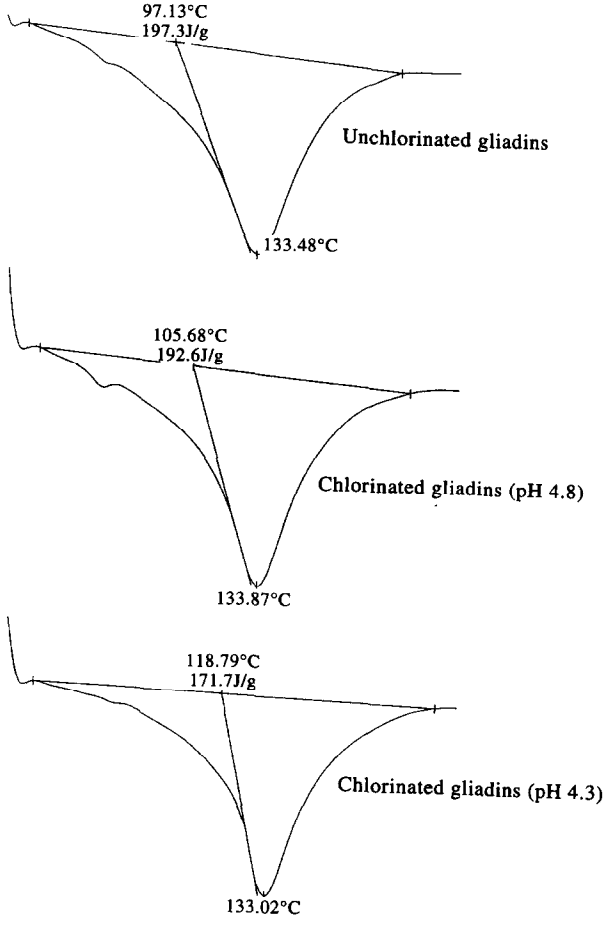

Fig. 3. Typical differential scanning thermograms of unchlorinated and chlorinated gliadin fractions.

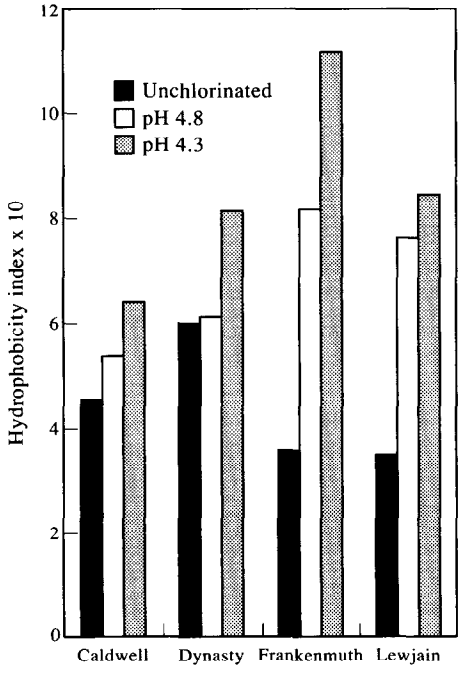

Fig. 4. Hydrophobicity indices of gliadins from unchlorinated and chlorinated flours.

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