Solvent Effects on the Molecular Structures of Crude Gliadins as Revealed by

Density and Ultrasound Velocity Measurements

by

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Abstract

Gliadin is associated with some of the functional properties of dough and the breadmaking suitability of flours. This study was undertaken to characterize the effects of solvents on the molecular structure of crude gliadins in terms of the physical properties of these proteins as assessed by ultrasound velocity and density measurements.

In the preliminary experiments, crude gliadins were extracted using modified Osborne fractionation from flours from four wheat cultivars (Glenlea, Katepwa, AC Reed and Genesis) and the ultrasound velocities of the crude gliadin serial dilutions were measured at 20 °C. The ultrasound velocity values of the crude gliadins in alcoholic solutions exhibited non-linearity with solution concentrations. Thus, fresh 70% (v/v) aqueous ethanol solutions made of 95% (v/v) ethanol and water were selected for further experiments to extract the crude gliadins from wheat flours.

For the solvent analyses, crude gliadins were extracted from Canada Western Red Spring (CWRS) wheat flour with 70% (v/v) aqueous ethanol solutions and then lyophilized. Lyophilized crude gliadins were dissolved in 70% (v/v) aqueous ethanol (EtOH) or 4 mM acetic acid (HAc) and the density and ultrasound properties were measured at 20 °C. Good linear relationships of density, ultrasound velocity and ultrasound attenuation with solution concentrations were found. Solvent and sonication effects on the crude gliadins were discussed in terms of the values of the partial specific volume, $\bar{v}^o$, and the partial specific adiabatic compressibility coefficient, $\bar{\beta}_s^o$, for crude gliadins. The ethanol soluble

crude gliadins had a larger partial specific volume, $\bar{v}^o$ (0.747 mL·g^{-1}), and larger partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ (10.27 ×10^{-6} bar^{-1}) than those for acidic soluble crude gliadins ($\bar{v}^o = 0.736$ mL·g^{-1}; $\bar{\beta}_S^o = 3.72$ ×10^{-6} bar^{-1}). These large values for the physical properties of ethanol soluble crude gliadins were thought to be evidence for the existence of complexes formed by some proteins (ethanol soluble LMW-glutenins and gliadins) and lipids in ethanol solutions and it was also found that the protein-lipid complexes were not destroyed by sonication treatment.

In the flour analyses, crude gliadins were extracted from wheat flours of different classes (Canada Western Red Spring, Canada Western Extra Strong and Soft White Spring) with 70% (v/v) aqueous ethanol solutions and freezed dried material was subsequently dissolved in 4 mM HAc. The density and ultrasound velocity and ultrasound attenuation were measured at 20 °C. Good linearity of density and ultrasound properties with solution concentration was found for the crude gliadins from the three different flours. There was no evidence showing that gliadins change with different wheat flours and cause different volume and compressibility properties of crude gliadins. However, the volume and compressibility properties were found to be related to the protein contents of the crude gliadin solutions, as well as the protein contents of the flours, and proteins had positive contributions to the volume and compressibility properties of acidic soluble crude gliadins and non-protein components had negative contributions.

A consistent set of physical parameters

Symbol	Quantity	Units
α	Thermal expansibility coefficient of a solution	K^{-1}
β	Compressibility coefficient of a solution	bar^{-1}
β_S	Adiabatic compressibility coefficient of a solution	bar^{-1}
β_{So}	Adiabatic compressibility coefficient of a solvent	bar^{-1}
$[\beta_S]$	Concentration increments of adiabatic compressibility coefficient of a solution	$mL{\cdot}mg^{-1}$
$\bar{\beta}_S^o$	Partial specific adiabatic compressibility coefficient of a solute (protein)	bar^{-1}
$\bar{\beta}_{SM}^o$	Partial specific intrinsic adiabatic compressibility coefficient of a protein	bar^{-1}
β_T	Isothermal compressibility coefficient of a solution	bar^{-1}
β_{To}	Isothermal compressibility coefficient of a solvent	bar^{-1}
$\bar{\beta}_T^o$	Partial specific isothermal compressibility coefficient of a solute (protein)	bar^{-1}
Φ_o	Apparent volumetric fraction of a solvent	-
γ_{cha}	Unit compressibility contributions of charged protein surface area	$cm^3mol^{-1}bar^{-1}\text{Å}^{-2}$
γ_{non}	Unit compressibility contributions of non-polar protein surface area	$cm^3mol^{-1}bar^{-1}\text{Å}^{-2}$

γ_{pol}	Unit compressibility contributions of polar protein surface area	$cm^3 mol^{-1} bar^{-1} Å^{-2}$
θ_{acc}	Total solvent accessible surface area of a mole of protein	$Å^2$
θ_{cha}	Solvent accessible surface area for charged hydrations of a mole of protein	$Å^2$
θ_{non}	Solvent accessible surface area for non-polar hydrations of a mole of protein	$Å^2$
θ_{pol}	Solvent accessible surface area for polar hydrations of a mole of protein	$Å^2$
ρ	Density of a solution	$mg·mL^{-1}$
ρ_o	Density of a solvent	$mg·mL^{-1}$
ρ_{sp}	Specific density	-
$[\rho]$	Concentration increments of density of a solution	$mL·mg^{-1}$
$[\rho]_{sp}$	Slope of the plot of ρ_{sp} against concentration	$mL·mg^{-1}$
a	Ultrasound attenuation of a solution	m^{-1}
a_o	Ultrasound attenuation of a solvent	m^{-1}
a_{sp}	Specific ultrasound attenuation	-
$[a]_{sp}$	Slope of the plot of a_{sp} against concentration	$mL·mg^{-1}$
c	Solute (protein) concentration in a solution	$mg·mL^{-1}$
C_P	Specific heat capacity of a solution under constant pressure	$J·kg^{-1}K^{-1}$
f	Frequency of an ultrasonic wave	Hz

ΔG_{unf}	Free energy of protein unfolding in water	$J \cdot g^{-1}$
K	Compressibility of a solution	$cm^3 bar^{-1}$
K_S	Adiabatic compressibility of a solution	$cm^3 bar^{-1}$
K_{So}	Adiabatic compressibility of a solvent	$cm^3 bar^{-1}$
$\bar{K}_S$	Apparent specific adiabatic compressibility	$cm^3 g^{-1} bar^{-1}$
$\bar{K}_S^o$	Partial specific adiabatic compressibility	$cm^3 g^{-1} bar^{-1}$
$\bar{K}_M^o$	Intrinsic contribution to the partial specific adiabatic compressibility	$cm^3 g^{-1} bar^{-1}$
$\Delta \bar{K}_I^o$	Hydration contribution to the partial specific adiabatic compressibility	$cm^3 g^{-1} bar^{-1}$
$\Delta \bar{K}_S^o$	Changes in partial specific adiabatic compressibility	$cm^3 g^{-1} bar^{-1}$
m	Mass of a solution	g
M	Protein molecular weight	$g \cdot mol^{-1}$
p	Pressure	Pa
$|q_i|$	Absolute partial charge	e (=1.602192 X10^{-19} C)
R	Ideal gas constant	(= 8.3143) $J \cdot K^{-1} mol^{-1}$
T	Absolute temperature	K
u	Ultrasound velocity of a solution	$m \cdot s^{-1}$
u_o	Ultrasound velocity of a solvent	$m \cdot s^{-1}$
u_{sp}	Specific ultrasound velocity	-
$[u]$	Concentration increments of ultrasound velocity of a solution	$mL \cdot mg^{-1}$

$[u]_{sp}$	Slope of the plot of u_{sp} against concentration	mL·mg^{-1}
v	Volume of a solution	mL
v_o	Volume of a solvent	mL
$\bar{v}$	Apparent specific volume	mL·g^{-1}
$\bar{v}^o$	Partial specific volume	mL·g^{-1}
$\bar{v}_C^o$	Volume of the cavity created in a solvent by a unit mass of a protein	mL·g^{-1}
$\bar{v}_I^o$	Hydration contribution to the partial specific volume	mL·g^{-1}
$\bar{v}_M^o$	Partial specific intrinsic volume of a protein	mL·g^{-1}
$\bar{v}_T^o$	Thermal volume of a unit mass of protein due to thermal molecular vibrations of the protein	mL·g^{-1}
v_W	Van der Waals volume of all constituent atoms in a unit mass of protein	mL·g^{-1}
v_v	Volume of the voids inside a unit mass of protein	mL·g^{-1}

Abbreviations used

CWES   Canada Western Extra Strong

CWRS   Canada Western Red Spring

EtOH   ethanol

HAc   acetic acid

HCl   hydrochloric acid

HMW   high molecular weight

LMA   lipid mediated aggregates

LMW   low molecular weight

NaCl   sodium chloride

SWS   Soft White Spring

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1. Introduction

Wheat is one of the most important food crops and is globally produced. Wheat flours can form viscoelastic dough by mixing with water and the dough can retain gas and be transformed into soft bread through heating. This makes the mixing properties and breadmaking suitability of wheat flours popular topics for cereal scientists.

Although the functionalities of wheat flours are related to many flour properties, it is generally considered that the gluten proteins are the key factor to breadmaking suitability of wheat flours (MacRitchie, 1992; Weegels *et al.*, 1996; Veraverbeke and Delcour, 2002; Suchy *et al.*, 2003). Finney and Barmore (1948) found a strong relationship between total protein content and the loaf volume of wheat flours. However, this relationship was insufficient to explain the inherent qualities of wheat flours of different grades (Suchy *et al.*, 2003). Subsequently, many researchers established relationships between flour quality and gliadins (Hoseney *et al.*, 1969a; Preston and Tipples, 1980; Békés *et al.*, 1992; Khatkar *et al.*, 2002), glutenins (Gupta *et al.*, 1995; Beasley *et al.*, 2002) and the ratio of gliadins to glutenins (Gupta *et al.*, 1992).

The gliadins, which comprise about 50% of the gluten proteins, are associated with some of the functional properties of dough and the breadmaking suitability of flours (MacRitchie, 1987; Fido *et al.*, 1997). However, there is a considerable debate on the functionalities of flour gliadins. Some researchers reported positive effects of gliadins on loaf volume (Hoseney *et al.*, 1969a; Preston and Tipples, 1980; Khatkar *et al.*, 2002) whereas others stated negative effects of gliadins

(MacRitchie, 1987). Different conclusions might arise from contamination from other material, e.g., low-molecular-weight glutenins and non-protein materials in gliadin extracts, as well as the structural change of gliadins during extraction procedures (Weegels *et al.*, 1994; Fido *et al.*, 1997; Khatkar *et al.*, 2002).

It is well known that the properties of solvents have effects on protein structures and conformations (Damodaran, 1996). Proteins have net charges in solutions where the pH values are not close to their isoelectric points (pI) which increase the intra- and inter-molecular repulsive electrostatic forces and these forces increase the protein solubility in the solutions and favor the unfolding of the protein molecules (Goto *et al.*, 1990; Damodaran, 1996). Organic solvents (e.g., ethanol) in water decrease the hydrophobic interactions of protein molecules by solubilising the non-polar side chains of proteins (Damodaran, 1996). Salts, at low concentrations (ionic strength $\leq$ 0.2), neutralize the net charges on the molecular surface of a protein and stabilize the protein native structures. This electrostatic interaction between salts and proteins is not related to the properties of the salts (Damodaran, 1996). However, at high concentrations (ionic strength > 1), salts have different effects on protein solutions, i.e., "salting-in" and "salting-out" (Damodaran, 1996). Although the principle of these two effects is not completely clear, it has been proposed that the chaotropic (salting-in) salts disturb the protein hydration shells and interact with proteins strongly, whereas the non-chaotropic (salting-out) ones interact with bulk water molecules strongly and protect the protein stability (Arakawa and Timasheff, 1984; Damodaran, 1996).

The partial specific adiabatic compressibility coefficient is a thermodynamic

parameter of a protein and is experimentally obtained from density and ultrasound velocity measurements for protein solutions (Sarvazyan, 1991). This parameter is widely used in studies on proteins including protein dynamics and enzyme functions because it provides information on protein structures and conformations (Chalikian *et al.*, 1995; Gekko, 2002). Besides, the partial specific adiabatic compressibility coefficient of a protein provides a new and powerful insight into the interaction of protein with solvent molecules in a solution (Townsend and Nakai, 1983; Gekko and Yamagami, 1991; Chalikian *et al.*, 1995; Chalikian *et al.*, 1996).

There does not appear to have been studies on gliadins in terms of the partial specific adiabatic compressibility coefficient, in spite of its ability to provide information on protein conformation and protein interactions with solvent molecules. Therefore, this parameter was determined for gliadins in different solvents. It is recognized that the conformation and the behaviour of dilute solutions of gliadins is very different than concentrated solutions of gliadins as found in dough. Nevertheless, the insights gained from studies under ideal solvent conditions may elucidate conformational structures of gliadins of relevance to dough quality.

The first objective of this study was to determine the behavior of alcoholic extracted crude gliadins from hard red spring wheat flours in two solvents, i.e., 70% (v/v) aqueous ethanol and 4 mM acetic acid. From the partial specific adiabatic compressibility coefficients of crude gliadins, deductions were made on the properties of lipid-protein complexes in ethanol soluble gliadin-rich fractions.

Complexes formed by gliadins and ethanol soluble LMW-glutenins and lipids were found in aqueous alcoholic extracts of wheat flours (Békés *et al.*, 1983a; Békés *et al.*, 1983b; Zawistowska *et al.*, 1984; McCann *et al.*, 2009) and were reported to have positive effects on flour breadmaking suitability (Békés *et al*., 1992).

Another objective is to determine whether the properties of gliadins vary with flours of different qualities. In this step, alcoholic extracted crude gliadins from flours of a range of quality are dissolved in 4 mM acetic acid. The concentration increments of density and ultrasound velocity of these solutions are determined and compared with each other. Based on the comparison, the partial specific adiabatic compressibility coefficients are estimated and relationships between flour baking suitability and the physical properties of crude gliadins are explored.

2. Literature Review

2.1. Volumetric and Compressible Properties of Protein Solutions

2.1.1. Volume of Proteins

2.1.1.1. Partial Specific Volume of Protein Molecules

Volume has a very easy definition as how much three-dimensional space a substance occupies. However, this simple parameter provides not only the macroscopic information of an object but also even more at the molecular level. To obtain such information on molecular properties of solutes in a solution the so-called apparent specific and partial specific volumes are introduced.

It should be clarified here that apparent molar quantities, indicating changes in properties of a solution caused by adding one mole of a solute, are usually used to understand the solute properties in a solution, but in the studies of protein solutions, apparent specific quantities representing changes resulting from unit mass rather than unit mole of solute, are preferred for convenient comparisons of proteins with different molecular weights. Generally, apparent specific quantities are calculated from the apparent molar ones divided by the molecular weight, M (Kharakoz and Sarvazyan, 1993; Chalikian *et al.*, 1994a; Chalikian *et al.*, 1994b; Taulier and Chalikian, 2002).

The apparent specific volume of a protein in a solution, expressed as $\bar{v}$, shows the change in volume of the solution resulting from a unit change in the solute mass (Moore, 1976). Thus, it is defined as

$$\overline{v} \equiv \frac{v - v_o}{cv} \tag{1}$$

where v and v_o are the volumes of the solution and the solvent before the solute is added in; c is the concentration of the solute, or protein, in the solution.

Experimentally, $\overline{v}$ is determined from the density values of the solution and the solvent and the protein concentration of the solution as equation 2 and is found to be independent of the protein concentration in dilute solutions:

$$\overline{v} = \frac{1 - (\rho - c)/\rho_o}{c} \tag{2}$$

where ρ and ρ_o are densities of the solution and the solvent, respectively. The expression $(\rho - c)/\rho_o$ is also defined as the apparent volumetric fraction of the solvent, which numerically equals the term v_o/v and can be denoted as Φ_o (Galema and Hoiland, 1991; Sarvazyan, 1991; Povey, 1997; Chalikian, 1998).

To approximate the properties of the ideal isolated protein molecules, which have no intermolecular interactions, the apparent specific volume is extrapolated to the limit of zero protein concentration and the partial specific volume of the protein molecule in the solution, $\overline{v}^o$, is obtained as equation 3 (Gekko and Noguchi, 1974; Millero *et al.*, 1976; Gekko and Noguchi, 1979; Gekko and Hasegawa, 1986; Gekko and Hasegawa, 1989; Gekko and Yamagami, 1991; Kamiyama and Gekko, 2000; Gekko *et al.*, 2003; Gekko *et al.*, 2004; Chalikian *et al.*, 1993; Blandamer *et al.*, 2001; Taulier *et al.*, 2005).

$$\overline{v}^o \equiv \lim_{c \to 0} \overline{v} = \lim_{c \to 0} \frac{1 - \Phi_o}{c} \tag{3}$$

2.1.1.2. Interpretation of Protein Partial Specific Volume

Since the partial specific volume gives information on ideally isolated protein molecules the parameter is interpreted microscopically. According to the scaled particle theory, a two-step process of the dissolution of a solute is proposed: 1. a cavity is created in the solvent large enough to accommodate the solute molecule; and 2. the solute molecule is placed into the cavity and the solute molecules interact with the solvent (Pierotti, 1976; Chalikian and Breslauer, 1996), so that the partial specific volume, $\bar{v}^o$, of a protein is considered to consist of three contributions:

$$\bar{v}^o = \bar{v}_C^o + \bar{v}_I^o + \beta_{To}RT/M \tag{4}$$

In equation 4, the subscript $_C$ indicates the volume of the cavity created by a unit mass of a protein in the solvent. The subscript $_I$ means the "interaction volume" representing the changes in solvent volume due to the interaction (hydration) between a unit mass of solute molecules and the solvent, which induces alterations and rearrangements of the solvent molecules; β_{To} is the isothermal compressibility coefficient of the solvent; R is the ideal gas constant and M and T are the molecular weight of the proteins and the absolute temperature, respectively. The term $\beta_{To}RT/M$ represents the effects on the solvent volume resulting from changes in the pressure of the system due to the kinetic contributions of the unit mass of the solute. For macromolecules such as protein molecules $\beta_{To}RT/M$ is small and can be neglected (Stillinger, 1973; Kharakoz, 1992; Chalikian and Breslauer, 1996; Chalikian, 2003).

Because there are two terms involved in the cavity volume: 1. the intrinsic volume of the solute, when expressed on a partial specific volume basis, $\overline{V}_M^o$, representing the geometric volume occupied by a unit mass of the solute molecules; and 2. the specific thermal volume, $\overline{V}_T^o$, representing an "empty" domain around the unit mass of protein molecules due to thermal molecular vibrations of the proteins and the solvent, equation 4 is improved as

$$\overline{V}^o = \overline{V}_M^o + \overline{V}_T^o + \overline{V}_I^o + \beta_{To} RT / M \tag{5}$$

Generally, the contribution of hydration, $\overline{V}_I^o$, depends on the chemical nature of the solvent accessible surface of a solute and is deemed to be a negative contribution to the partial specific volume, $\overline{V}^o$, of a protein because the solvent molecules in the hydration shells have smaller partial specific volumes compared with the bulk ones (Gekko and Noguchi, 1979; Gekko and Hasegawa, 1986; Gekko and Hasegawa, 1989; Gekko and Yamagami, 1991; Kharakoz, 1992; Chalikian and Breslauer, 1996; Chalikian *et al.*, 1996; Chalikian, 1998; Chalikian and Breslauer, 1998; Murphy *et al.*, 1998; Chalikian, 2001; Gekko, 2002; Valdez *et al.*, 2001; Chalikian, 2003; Gekko *et al.*, 2003; Gekko *et al.*, 2004; Bano and Marek, 2006).

2.1.1.3. Intrinsic Volume and Interior Atomic Packing of Proteins

The partial specific intrinsic volume of a protein, $\bar{v}_M^o$, equals to the sum of the Van der Waals volume of all constituent atoms, v_W, plus the total volume of the voids inside the protein molecule, v_V. The voids inside the protein molecule result from the imperfect packing of the atoms of a protein. The imperfect packing is due to the irregular shapes of the molecular groups on the proteins or their secondary strucutures and the repulsion among these groups. To describe the interior imperfect packing of the protein molecules the packing density is introduced as the ratio of the protein's Van der Waals volume to its intrinsic volume. As far as the partial specific intrinsic volume is concerned, the packing density is calculated as $v_W/\bar{v}_M^o$. From this perspective, perfect packing occurs when the packing density is 1. However, the values of the packing density of different globular proteins are smaller than 1 but similar, having an average value of about 0.75, indicating similar solid-like interior structures of globular proteins (Chalikian *et al.*, 1996; Taulier and Chalikian, 2002; Mori *et al.*, 2006). Chalikian *et al.* (1996) proposed that the average contribution of $\bar{v}_M^o$ to $\bar{v}^o$ is about 90%. The remaining parts in the right-hand side of equation 5 totally account 10% towards $\bar{v}^o$, of which only the interaction volume, $\bar{v}_I^o$, is related to the hydration states of protein molecules. Thus, volumetric properties are not sensitive enough on their own for analysis of protein hydration, and so the compressible properties of proteins and protein solutions are introduced.

2.1.2. Compressibility of Proteins and Their Solutions

2.1.2.1. Compressibility and Compressibility Coefficient

The compressibility and the compressibility coefficient are introduced to indicate the compressible properties of a system by showing the relationship between volume and pressure. Compressibility, K, is defined as the pressure dependence of the volume:

$$K \equiv -\frac{\partial v}{\partial p} \qquad (6)$$

where v is the instantaneous volume and p is the instantaneous pressure. According to the definition of compressibility, the compressibility coefficient, β, which is used more widely, can be defined as the pressure dependence of the relative volume (Gavish *et al*., 1983; Bloor *et al*., 1983; Cooper, 1984; Heremans and Smeller, 1998; Blandamer *et al*., 2001; Taulier *et al*., 2005):

$$\beta \equiv \frac{K}{v} = -\frac{1}{v} \times \frac{\partial v}{\partial p} \qquad (7)$$

However, equations 6 and 7 just provide very rough definitions because in thermodynamics the parameters are expressed as adiabatic or isothermal depending on the environment with constant entropy or constant temperature, respectively. Thus, the adiabatic, β_S, and isothermal, β_T, compressibility coefficients are applied. The thermodynamic relation between them is known as

$$\beta_T = \beta_S + \frac{\alpha^2 T}{\rho C_p} \qquad (8)$$

where the subscripts $_T$, $_S$ and $_p$ represent constant temperature, constant entropy,

and constant pressure, respectively; the letters T, ρ and C_p represent the absolute temperature, the density and the specific heat capacity of the system under constant pressure, respectively. The letter α is the thermal expansibility coefficient of the solution and is defined as

$$\alpha \equiv \frac{1}{v} \times \left(\frac{\partial v}{\partial T} \right)_p \tag{9}$$

For aqueous solutions the difference between β_S and β_T is small so that it does not exceed a few percent because of the large value of C_p and the small value of thermal expansibility coefficient for water (Millero *et al.*, 1976; Hoiland, 1980; Gekko and Hasegawa, 1986; Gekko and Hasegawa, 1989; Sarvazyan, 1991; Tikhonov *et al.*, 1995; Povey, 1997; Heremans and Smeller, 1998; Taulier and Chalikian, 2002).

2.1.2.2. Ultrasound Velocity and Adiabatic Compressibility Coefficient of Liquids

For liquid systems, the adiabatic compressibility coefficient, β_S, is practically determined based on measurements of ultrasound velocity and density, which are believed to be the only direct way to evaluate the adiabatic compressibility coefficient (Sarvazyan, 1991).

Low intensity ultrasound is utilized widely in industry. Successful evaluation of the properties of opaque materials makes the technique a good supplement for light-based determination. Ultrasound has a non-destructive effect on samples during detection, and this is another desirable advantage (Awad, 2004; Coupland, 2004; Scanlon, 2004). Owing to fantastic developments in ultrasonic techniques,

accurate and precise measurement of ultrasound velocity is achieved, which permits more utilization of ultrasound technology in research fields as diverse as the detection of particle size, distribution and concentration of particles in suspensions as well as determination of the adiabatic compressibility coefficient with high accuracy.

Ultrasounds are sonic waves with frequencies above 20,000 Hz. Ultrasound propagating in fluids occurs as longitudinal waves. Longitudinal waves propagate with all the points in the medium moving back and forth in the direction in which the waves travel, such as the vibrations of a spring. Transverse waves, for instance those on a string, make the points in the medium move perpendicularly to the direction of their propagation (as shown in Figure 2.1). In the propagation of longitudinal waves, there are no alternate crests or troughs but alternate compressions and rarefactions in the medium during propagation (Morse and Ingard, 1968). When the fluid media are compressed or expanded by ultrasound waves and the particles in the media are disturbed, the corresponding restoring forces keeping the particles of the medium in their original positions are simply the changes of the pressure.
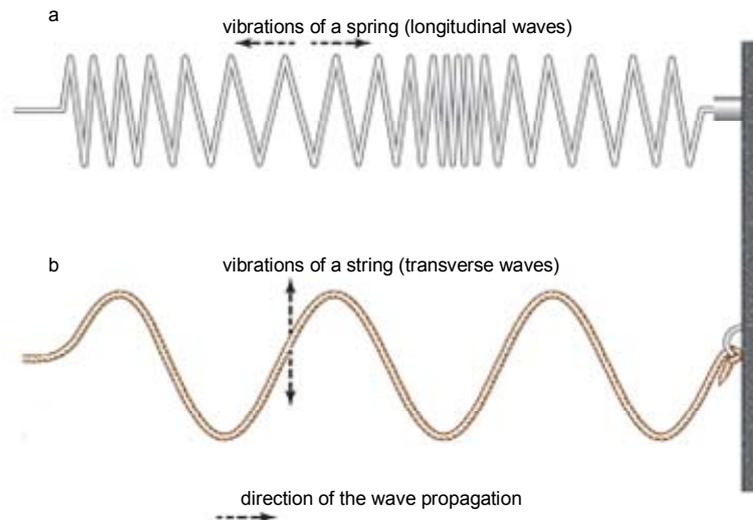
Figure 2.1 (a) longitudinal waves on a spring make the particles in a medium move back and forth in the direction of their propagation; (b) transverse waves on a string make the particles in a medium vibrate perpendicular to the direction of wave propagation (Public domain).

It is found experimentally that there is no exchange of thermal energy between two neighbouring particles during sonic wave propagation in homogeneous fluids (Kinsler *et al.,* 1982). In this case, it is the entropy but not the temperature of the fluid that remains almost constant. The adiabatic sound propagation is therefore dependent on the adiabatic properties of the medium in which the sonic waves propagate.

The particles of the media are vibrating at different pressures in the sound field and the sound velocity is a simple function of the pressure derivative of density (Sarvazyan, 1991):

$$u^2 = \left(\frac{\partial p}{\partial \rho} \right)_S \tag{10}$$

where u, p and ρ represent the sound velocity, pressure and density of the solution, respectively, and the subscript s means under constant entropy. From $\rho = m/v$, equation 10 is improved as

$$\frac{1}{u^2} = \left[\frac{\partial(m/v)}{\partial p}\right]_s = \frac{1}{v} \times \left(\frac{\partial m}{\partial p}\right)_s - \frac{m}{v^2} \times \left(\frac{\partial v}{\partial p}\right)_s$$

where m is the mass of the solution and is independent of pressure, p, so,

$$\frac{1}{u^2} = -\frac{\rho}{v} \times \left(\frac{\partial v}{\partial p}\right)_s$$

Thus, from equation 7, the value of β_S is obtained as

$$\beta_S = \frac{1}{\rho u^2} \tag{11}$$

Equation 11 is the very famous Laplace equation for the calculation of the adiabatic compressibility coefficient from density and sound velocity data (Gekko and Noguchi, 1974; Gekko and Noguchi, 1979; Sarvazyan *et al.*, 1979; Chavez *et al.*, 1985; Gekko and Hasegawa, 1986; Sarvazyan, 1991; Chalikian *et al.*, 1992; Kharakoz and Sarvazyan, 1993; Chalikian *et al.*, 1994a; McClements, 1995; Tikhonov *et al.*, 1995; Paci and Marchi, 1996; Hianik *et al.*, 1997; Pinfield and Povey, 1997; Povey, 1997; Heremans and Smeller, 1998; Povey, 1998; Burakowski and Gliński, 2007; Raman *et al.*, 2007).

The Laplace equation shows that there is a strong dependence of sound propagation on the physical properties of the medium. When sound waves are passing through liquid media it is the density and the adiabatic compressibility coefficient that are the physical properties of the fluid media that affect wave propagation. In physics the density and the adiabatic compressibility coefficient

are used to provide information on the mass and the elasticity of the particles of the media which are vibrating during sonic wave propagation (Povey, 1997). From a straightforward view, vibrating particles with large mass and elasticity have inertia and cushioning actions, making them more difficult to move when pressure is applied.

2.1.2.3. Partial Specific Adiabatic Compressibility Coefficient

The adiabatic compressibility coefficient, β_S, calculated from the Laplace equation gives information on the properties of the liquid system (Townsend and Nakai, 1983; Gekko and Yamagami, 1991; Awad, 2004). However, this parameter is not enough for determining the properties of the solute, for instance proteins dissolved in a solvent, and the concept of partial specific compressibility coefficient is introduced.

Like the application of the partial specific volume of the proteins in a solution, the so-called partial specific compressibility coefficient is introduced for understanding the compressible or elastic properties of ideally isolated protein molecules in solution (Millero *et al*., 1976; Chalikian *et al*., 1993; Chalikian *et al*., 1994a). The partial specific compressibility coefficient can be adiabatic or isothermal, depending on the conditions under which it is measured. Based on ultrasound velocity and density measurements, the partial specific adiabatic compressibility coefficient is obtained.

According to the definition of compressibility coefficient (equation 7), the partial specific adiabatic compressibility coefficient, $\overline{\beta}_S^o$, is expressed as:

$$\overline{\beta}_S^o = -\frac{1}{\overline{v}^o} \times \left(\frac{\partial \overline{v}^o}{\partial p}\right)_S \qquad (12)$$

Equation 12 illustrates that the partial specific adiabatic compressibility coefficient, $\overline{\beta}_S^o$, represents the dependence of partial specific volume, $\overline{v}^o$, of the solute on pressure, p, under adiabatic conditions (constant entropy, $_S$) and it is rewritten as

$$\overline{\beta}_S^o = \frac{\overline{K}_S^o}{\overline{v}^o} \qquad (13)$$

where $\overline{K}_S^o$ is the partial specific adiabatic compressibility. Just as the apparent specific volume of a protein is defined by equation 1, the apparent specific adiabatic compressibility, $\overline{K}_S$, is defined as

$$\overline{K}_S \equiv \frac{K_S - K_{So}}{cv} \qquad$$

where K_S is the adiabatic compressibility of a solution and the subscript $_o$ indicates the parameters of the bulk solvent molecules.

According to the definition of apparent and partial quantities, the partial specific adiabatic compressibility, $\overline{K}_S^o$, is expressed as

$$\overline{K}_S^o = \lim_{c \to 0} \overline{K}_S = \lim_{c \to 0} \frac{K_S - K_{So}}{cv} \qquad (14)$$

From equation 7 and equation 13, equation 14 is rewritten as

$$\overline{\beta}_S^o \times \overline{v}^o = \lim_{c \to 0} \frac{\beta_S \times v - \beta_{So} \times v_o}{cv} \qquad (15)$$

Experimentally, the adiabatic compressibility coefficients of solution and

solvent, $\beta_S = \dfrac{1}{\rho u^2}$ and $\beta_{So} = \dfrac{1}{\rho_o u_o^2}$ (equation 11), the apparent volumetric

fraction of solvent, $\Phi_o = v_o/v = \dfrac{\rho - c}{\rho_o}$, and the partial specific volume of proteins,

$\bar{v}^o = \lim\limits_{c \to 0} \dfrac{1 - \Phi_o}{c}$ (equation 3), are determined based on the measurements of

ultrasound velocity, u, and density, ρ, of solutions with known concentrations, c

and those u_o and ρ_o of a solvent. Consequently, like the partial specific volume,

$\bar{v}^o$, the partial specific adiabatic compressibility coefficient of a protein in the

solution, $\bar{\beta}_S^o$, is also obtained via the zero concentration extrapolation method.

Thus, equation 15 gives (Gekko and Noguchi, 1974; Gekko and Noguchi, 1979;

Gekko and Hasegawa, 1986; Gekko and Hasegawa, 1989; Gekko and Yamagami,

1991; Paci and Marchi, 1996; Kamiyama and Gekko, 2000; Gekko *et al*., 2003;

Gekko *et al*., 2004)

$$\bar{\beta}_S^o = \left(\frac{\beta_{So}}{\bar{v}^o} \right) \times \lim_{c \to 0} \frac{\beta_S/\beta_{So} - \Phi_o}{c} \tag{16}$$

Besides the conventional approach to evaluate the partial specific adiabatic

compressibility coefficient of proteins, Sarvazyan (1991) proposed an alternative

one. Firstly, the so-called "concentration increments" are defined for density, ρ,

ultrasound velocity, u, and adiabatic compressibility coefficient, β_S, as

$$[\rho] = \lim_{c \to 0} \frac{\rho - \rho_o}{\rho_o c} \tag{17}$$

$$[u] = \lim_{c \to 0} \frac{u - u_o}{u_o c} \tag{18}$$

$$[\beta_S] = \lim_{c \to 0} \frac{\beta_S - \beta_{So}}{\beta_{So}c} \tag{19}$$

where the quantities in square brackets are concentration increments; c is the concentration of the protein solution; the subscript o indicates the properties of the solvent. The relationship between these relative increments is obtained by differentiating the Laplace equation (equation 11) as

$$[\beta_S] = -2[u] - [\rho] \tag{20}$$

Further, the apparent specific adiabatic compressibility of a protein, $\bar{K}_S$, is obtained via

$$\bar{K}_S = \beta_{So} \times \left(\bar{v} + [\beta_S] \right) \tag{21}$$

and the partial specific adiabatic compressibility, $\bar{K}_S^o$, is obtained by performing zero concentration extrapolation on $\bar{K}_S$.

In Sarvazyan's (1991) approach the measurement error depends on the precision of evaluation of only the differences between solution and solvent but not the absolute values of density and ultrasound velocity in both the solution and the solvent. He stated that such differential measurements were one or two orders of magnitude more precise than the absolute measurements. Besides, the concentration increment of the compressible properties also provides important information on the molecular structure of proteins in solution (Sarvazyan, 1991; Chalikian *et al*., 1992; Chalikian *et al*., 1993; Chalikian *et al*., 1994a; Chalikian *et al*., 1994b; Povey, 1997; Kharakoz and Bychkova, 1997; Chalikian, 1998; Hianik *et al*., 1997; Taulier *et al*., 2005).

Based on the value of partial specific adiabatic compressibility coefficient, $\overline{\beta}_S^o$, the partial specific isothermal compressibility coefficient, $\overline{\beta}_T^o$, is determined from measurements of the expansibility coefficients and the specific heat capacity of the solvent and proteins (Mori *et al*., 2006). The partial specific isothermal compressibility coefficient is used in studies of the protein dynamics for the measurements of the volume fluctuation of protein molecules in terms of statistical thermodynamics (Cooper, 1976; Eden *et al*., 1982; Gekko and Hasegawa, 1986; Chalikian *et al*., 1995; Taulier and Chalikian, 2002; Chalikian, 2003), which is widely used in studies of enzyme functionality (Gekko and Hasegawa, 1986; Gekko *et al*., 1996; Kamiyama and Gekko, 2000; Gekko, 2002). Since this topic is not involved in this research, it is not introduced here.

Kharakoz and Sarvazyan (1993) found that the period of ultrasound propagation in a solution was not short enough to exclude all the heat exchange between solute molecules, hydration shells and the solvent in their experiment using ultrasound with a frequency at 7.2 MHz. They pointed out that the ultrasound velocity only provided the pseudoadiabatic compressibility coefficient, which is an intermediate value between the adiabatic and the isothermal ones. Based on theoretical calculations, Nölting (1995) proposed that for common proteins (less than 100 kDa in molecular weight) in dilute aqueous solutions (less than 2% protein concentration) at a temperature lower than 25 °C, ultrasound measurements performed with a frequency less than 10 MHz on the protein solutions provide values that are close to isothermal conditions rather than adiabatic conditions. The reason is that the distance between the protein

molecule and its hydration shell is relatively small compared with the ultrasonic wavelength, and so thermal equilibrium between protein and water molecules can be reached in the nanosecond time scale (Nölting *et al.*, 1997). Therefore, this time scale is too short for ultrasound waves with frequencies less than 10 MHz to exclude the effect of heat exchange (Pfeiffer *et al.*, 2008). Thus, the measured partial specific adiabatic compressibility coefficients of globular proteins might be $3 \sim 4 \times 10^{-6}$ bar^{-1} bigger than the true values. For instance, the partial specific adiabatic compressibility coefficient of haemoglobin was reported as 11×10^{-6} bar^{-1} (Gekko and Hasegawa, 1986) but the true value might be about 8×10^{-6} bar^{-1} as predicted by Nölting (1995). However, the theoretical treatment of Nölting (1995) was disputed by Gekko *et al.* (2009), who directly determined the partial specific adiabatic and isothermal compressibility coefficients of BSA at different temperatures using ultrasound velocimetry and oscillating densitometry, respectively. From the results, Gekko *et al.* (2009) found that the directly measured partial specific isothermal compressibility coefficient of BSA was distinctly larger than the adiabatic one determined from ultrasound velocimetry at low temperatures (5 ~ 20 °C), which contradicted the prediction that they should be close to each other below 25 °C (Nölting, 1995). It should be noted here, that there are still unknown factors affecting both the experiments and the theories of protein compressibility properties (Gekko *et al.*, 2009), and in this thesis, the compressibility coefficients obtained from ultrasound velocimetry are considered to occur under adiabatic conditions according to practice in most of the related studies (*Section 2.1.2.2*).

2.1.3. Compressible Properties and Protein Structure

As physical parameters, the compressibility and compressibility coefficient are related to the structure of the molecule. When examining the structure of a protein molecule, a number of different structural levels are apparent (Gavish *et al.*, 1983; Gekko *et al.*, 1996). It would be expected that each of the various structural levels would affect the compressibility of the protein molecule. For example, the number of hydrophobic amino acids and the type of secondary structures are believed to contribute to the compressible properties of a protein (Gavish *et al.*, 1983; Gekko and Hasegawa, 1986). However, the primary and secondary structures of proteins are poorly correlated with the compressible properties of different proteins (Gavish *et al.*, 1983; Gekko and Yamagami, 1991; Dadarlat and Post, 2001). For instance, a high homology between hen egg lysozyme and bovine milk α-lactalbumin is found in the primary (45% homology) and the secondary (29-33% α-helix and 16-17% β-sheet) structures, but α-lactalbumin is highly compressible compared with lysozyme (Gekko and Yamagami, 1991). Thus, the compressibility properties of a protein are more strongly related to the tertiary structure (i.e., the manner of packing of secondary structures) of the protein molecule (Gekko and Hasegawa, 1986). As a consequence, the compressibility parameters are better understood in terms of the interior packing and the surface hydration properties of protein molecules.

2.1.3.1. Interpretation of Compressibility

Similar to the partial specific volumetric properties of proteins, the partial specific adiabatic compressibility, $\bar{K}_S^o = \bar{\beta}_S^o \times \bar{v}^o$, of proteins consists of the sum of an intrinsic contribution, $\bar{K}_M^o$, and hydration contributions, $\Delta \bar{K}_I^o$:

$$\bar{K}_S^o = \left(\bar{K}_M^o + \Delta \bar{K}_I^o \right)_S \tag{22}$$

where the subscripts M and I represent intrinsic molecular and hydration contributions (Kharakoz, 1991; Sarvazyan, 1991; Kharakoz and Sarvazyan, 1993; Chalikian *et al.*, 1994a; Chalikian *et al.*, 1994b; Chalikian and Breslauer, 1995; Chalikian *et al.*, 1995; Chalikian, 1998; Chalikian and Breslauer, 1998; Taulier and Chalikian, 2002). Since the intrinsic volume of a protein contains two parts, i.e., Van der Waals volume of all constituent atoms and the void volume inside the globular molecule (*Section 2.1.1.3*), and the constituent atoms are much less compressible than the void space for a protein, the intrinsic contribution, $\bar{K}_M^o$, is mainly a result of the void cavity due to imperfect atomic packing in the protein interior. The hydration contributions, $\Delta \bar{K}_I^o$, are caused by the difference between the compressibility properties of the water molecules in the hydration shells and in the bulk solvent. From equation 22, information about the molecular structure of proteins, including the intrinsic cores and hydration shells of the molecules is accessible based on the value of partial specific compressibility.

2.1.3.2. Protein Characteristics and Compressible Properties

2.1.3.2.1. Hydration of Proteins

Firstly, the protein surface should be understood clearly since the interaction of protein and solvent molecules occurs on the surface of proteins. Three different types of surfaces for a single simple molecule are shown in Figure 2.2: Van der Waals surface, solvent accessible surface and molecular surface. Such surfaces would exist on a protein molecule, but molecular size would obviously be much larger. The Van der Waals surface is exactly the sum of the area of atoms or groups on the surface of a molecule, as the boundary of shadow spheres and the blank space in Figure 2.2. The solvent accessible area is defined as the area traced out by the center of a probe sphere which represents the solvent molecule (considering water molecules as a sphere with a radius of 1.4 Å) as it is rolled over the molecule as indicated by the dashed line in Figure 2.2 (Connolly, 1983). The molecular surface, as shown by the solid line in Figure 2.2, contains two parts: the contact surface, which is the part of the Van der Waals surface contacting the solvent molecules; and the re-entrant surface, which connects the contact surfaces when one solvent molecule is contacting more than one atom of a protein (Connolly, 1983; Totrov and Abagyan, 1996; Hayryan *et al*., 2005). Consequently, the molecular surface (solid line in Figure 2.2) has the same shape as the solvent accessible surface (dashed line in Figure 2.2) but with a smaller size. By the solvent accessible area and the radius of the probe sphere (e.g. 1.4 Å for water molecules), the area of the molecular surface is calculated. Practically,

this complicated simulation and calculation are completed by a computer based on the X-ray crystallographic structure.
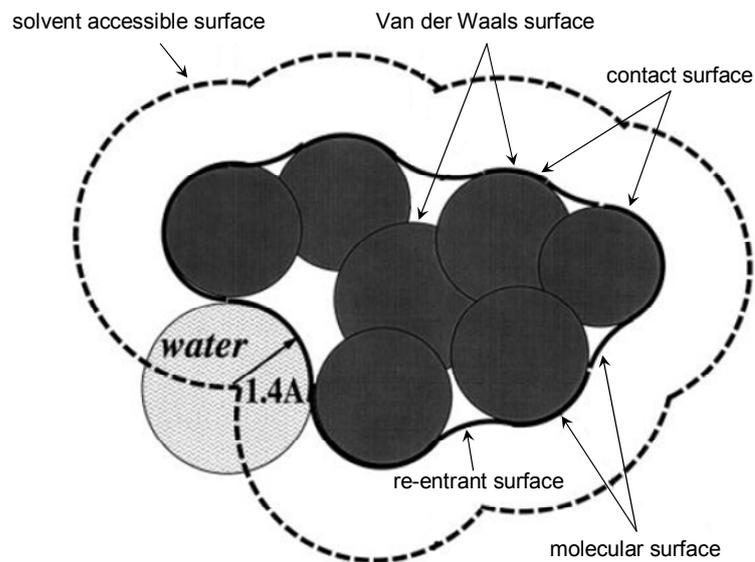
Figure 2.2 Different types of surfaces of a molecule used in molecular modeling and graphics. (Adapted from Journal of Structural Biology, v. 116, M Totrov and R Abagyan, The contour-buildup algorithm to calculate the analytical molecular surface, *p*. 139, Copyright (1996), with permission from Elsevier).

As expressed by equation 22, the hydration contribution of compressible properties of proteins is determined by the difference between the compressibility of molecules in hydration shells and in the bulk solvent. In an aqueous protein solution for instance, water molecules interacting with different surface groups of a protein, i.e., hydrations of charged, polar, and non-polar atoms or groups, have different compressible properties (Kharakoz, 1991; Chalikian *et al.*, 1996; Taulier

and Chalikian, 2002). Commonly, charged groups include the side-chain carboxyl groups of Asp and Glu, the guanidinium group of Arg, the side-chain amino group of Lys, the amino and carboxylate termini of the polypeptide chain, and the charged Ca^{2+} ions in α-lactalbumin and trypsin. The polar category includes atoms with an absolute partial charge $|q_i| > 0.3e$, excluding those atoms defined as charged. All other atoms are non-polar atoms (Dadarlat and Post, 2003; Dadarlat and Post, 2006).

Chalikian *et al*. (1996) improved equation 22 for globular proteins as

$$\bar{K}_S^o = \left(\bar{K}_M^o + \Delta \bar{K}_I^o \right)_S = \bar{\beta}_{SM}^o \times \bar{V}_M^o + \left(\gamma_{cha} \times \theta_{cha} + \gamma_{pol} \times \theta_{pol} + \gamma_{non} \times \theta_{non} \right) \Big/ M \quad (23)$$

where $\bar{\beta}_{SM}^o$ and $\bar{V}_M^o$ represent the partial specific intrinsic adiabatic compressibility coefficient and partial specific intrinsic volume, respectively (recall that "intrinsic" refers to the properties of only the globular protein molecules itself); γ ($cm^3mol^{-1}bar^{-1}Å^{-2}$) is the unit compressibility contribution of the solvent accessible surface area ($Å^2$) due to different hydrations (i.e., charged, polar and non-polar) on the protein solvent accessible surface; θ is the solvent accessible surface area ($Å^2$) of one mole of protein; M is protein molecular weight; and the subscripts *cha, pol* and *non* represent the charged, polar and non-polar groups on the protein molecular surface, respectively, which interact with water molecules differently.

Chalikian *et al*. (1996) calculated the unit compressibility contributions of charged, γ_{cha}, polar, γ_{pol}, and non-polar, γ_{non}, protein surface area from 18 °C to 55 °C in aqueous solutions. The negative values of unit compressibility contributions shown in Table 2.1 indicate that the interaction between the solvent

molecules and proteins contributes negatively to the protein compressibility properties (Gekko and Noguchi, 1979; Gekko and Hasegawa, 1986; Gekko and Hasegawa, 1989; Gekko and Yamagami, 1991; Kharakoz, 1991; Kharakoz and Sarvazyan, 1993; Chalikian and Breslauer, 1995; Chalikian *et al*., 1996; Paci and Marchi, 1996; Kharakoz, 1997; Heremans and Smeller, 1998; Gekko, 2002; Taulier and Chalikian, 2002; Valdez *et al*., 2001; Chalikian, 2003; Gekko *et al*., 2003; Marchi, 2003; Gekko *et al*., 2004; Bano and Marek, 2006; Dadarlat and Post, 2006; Mori *et al*., 2006).

Table 2.1 Values of coefficients γ_{cha}, γ_{pol}, and γ_{non} ($\times 10^{-6}$ cm^{3}mol^{-1}bar^{-1}Å^{-2}) in equation 22 (Chalikian *et al*., 1996).

Coefficients	18 °C	25 °C	35 °C	45 °C	55 °C
γ_{cha}	- 15 ± 10	- 15 ± 10	- 15 ± 10	- 15 ± 10	- 15 ± 10
γ_{pol}	- 66 ± 3	- 62 ± 3	- 60 ± 3	- 58 ± 3	- 57 ± 3
γ_{non}	- 13 ± 2	- 9 ± 2	- 6 ± 2	- 5 ± 2	- 4 ± 2

Chalikian *et al*. (1996) compared the unit compressibility contribution of charged, γ_{cha}, polar, γ_{pol}, and non-polar, γ_{non}, protein surfaces with those on low-molecular-mass compounds to illustrate the hydration properties of proteins.

Charged groups on the surfaces of proteins and low-molecular-mass compounds interact with water molecules through electrostatic interactions

(Fennema, 1996; Raschke, 2006). The unit compressibility contribution of a charged protein surface, γ_{cha}, being equal to $(-15 \pm 10) \times 10^{-6}$ $cm^3 mol^{-1} bar^{-1} Å^{-2}$, is similar to that for glycine $(-20 \times 10^{-6}$ $cm^3 mol^{-1} bar^{-1} Å^{-2})$ suggesting that proteins and low molecular mass compounds have similar hydration shells of charged groups as each other, where water molecules are strongly oriented in the electrostatic field of the charged groups (Kharakoz, 1991, Chalikian *et al*., 1993; Chalikian *et al*., 1996).

It has been pointed out that polar groups (with an absolute partial charge $|q_i| > 0.3e$ excluding charged groups) interact with water molecules directly through hydrogen bonds, whereas non-polar groups enhance hydrogen bonds among water molecules themselves, favouring the formation of clathrates around the non-polar groups by water molecules (Fennema, 1996; Raschke, 2006).

Chalikian *et al*. (1996) found that the unit compressibility contribution of polar protein surfaces, γ_{pol}, is almost three times more negative than that for an amino acid obtained by Kharakoz (1991). The disparity is explained in two aspects (Kharakoz, 1991; Chalikian *et al*., 1993; Chalikian *et al*., 1996): 1. when the polar groups on a protein surface are close enough (separated by three or less covalent bonds), such as those on the protein surface, the adjacent water molecule is postulated to simultaneously form hydrogen bonds with two or more neighbouring compatible polar groups; and 2. the hydrogen-bonded water molecules can form a network with the water molecules that are not contacting the protein surface through hydrogen bonds and thus extend the hydration shells of polar groups on the protein surface. The behaviour of water molecules in the hydration shells

surrounding the polar groups of proteins account for the negative values of γ_{pol} in Table 2.1, which suggest that the water molecules in the hydration shells surrounding polar groups (γ_{pol} is around -60×10^{-6} cm^{3}mol^{-1}bar^{-1}Å^{-2}) are even more incompressible than those around charged groups (γ_{cha} is around -15×10^{-6} cm^{3}mol^{-1}bar^{-1}Å^{-2}).

From comparisons of the unit compressibility contribution of non-polar surfaces of proteins, γ_{non}, with that of a non-branched chain of –CH$_2$– groups in low-molecular-mass compounds, Chalikian *et al*. (1996) found that the water molecules in the hydration shells surrounding the non-polar groups of proteins have similar behaviour as those around the non-polar groups of low-molecular-weight compounds at low temperatures (below 25 °C). However, when the temperature is increased, the water molecules adjacent to protein non-polar groups are less compressible than those around low-molecular-compounds. The possible explanation is that the hydrogen bonds among water molecules stabilizing the water clathrates around the non-polar groups weaken at high temperatures and these non-bonded water molecules form new hydrogen bonds with neighbouring polar groups on the protein surface (Kharakoz, 1991; Chalikian *et al*., 1993; Chalikian *et al*., 1994a; Chalikian *et al*., 1996).

For a better understanding of the relationship between the compressible properties of protein molecules and their hydration states, the relative surface areas of different groups and the partial specific adiabatic compressibility coefficient for 12 proteins are listed in Table 2.2 (Chalikian *et al*., 1996). Although

there is no strong correlation between the charged, polar and non-polar groups on the molecular surface and the compressible properties of the proteins, large non-polar surfaces tend to increase the values of the partial specific adiabatic compressibility coefficient. Thus, one can obtain a rough knowledge of the protein hydrophobicity from the partial specific adiabatic compressibility coefficient.

Table 2.2 The percentage of different solvent accessible surface areas (%) and the partial specific adiabatic compressibility coefficients ($\times 10^{-6}$ bar^{-1}) for 12 proteins (Chalikian $et\ al.$, 1996).

Protein	$\theta_{cha}\%$	$\theta_{pol}\%$	$\theta_{non}\%$	$\bar{\beta}_S^o$
Myoglobin	16.10	22.06	61.85	9.5
Hemoglobin	16.65	21.70	61.64	9.1
Ovalbumin	18.66	30.58	50.76	8.1
Pepsin	13.99	36.56	49.45	7.1
Trypsinogen	7.78	41.68	50.53	5.5
α-Chymotrypsinogen A	10.77	36.39	52.83	4.4
Cytochrome c	19.07	24.58	56.35	4.2
α-Chymotrypsin	11.62	37.46	50.92	3.9
α-Lactalbumin	21.45	25.97	52.58	3.8
Trypsin	8.70	40.70	50.59	2.4
Lysozyme	13.57	38.12	48.32	1.9
Ribonuclease A	13.65	35.36	50.99	1.3

2.1.3.2.2. Interior of Proteins

In Table 2.1, the hydration of charged, polar and non-polar groups on protein surface all have negative contributions to the compressibility properties of proteins. Therefore, we would expect hydration to lead to a negative value for $\overline{\beta}_S^o$. However, the globular proteins have positive values of $\overline{\beta}_S^o$ as shown in Table 2.2. The interior structures of proteins account for this positive contribution.

It has been reviewed in *Section 2.1.1.3* that the packing densities of different globular proteins are alike, having an average value of about 0.75, indicating similar imperfect packing of atoms in the interior of globular proteins (Richards, 1977; Chalikian *et al.*, 1996; Valdez *et al.*, 2001; Taulier and Chalikian 2002). This imperfect atomic packing inside proteins, which forms void space in the interior core of globular proteins, accounts for the positive compressible properties of proteins. It has been also considered that the partial specific intrinsic adiabatic compressibility coefficients, $\overline{\beta}_{SM}^o$, are alike for all globular proteins (Gekko and Noguchi, 1979; Kharakoz and Sarvazyan, 1993; Chalikian *et al.*, 1994a; Chalikian *et al.*, 1996; Marchi, 2003). Although this idea was disputed by Mori *et al.* (2006), who proposed that the partial specific intrinsic adiabatic compressibility coefficient varied with protein species according to the estimation from molecular dynamic simulations, it is widely considered that the average value of partial specific intrinsic adiabatic compressibility coefficient, $\overline{\beta}_{SM}^o$, of all globular proteins is $(25 \pm 1) \times 10^{-6}$ bar^{-1} and temperature independent (Chalikian and Breslauer, 1998; Kharakoz, 2000; Taulier and Chalikian, 2002; Chalikian, 2003).

2.1.3.3. Conformation and Compressible Properties of Proteins

The conformation of a globular protein is understood to occur in four states: native (N), compact intermediate (CI), partial unfolded (PU) and fully unfolded (FU) (Chalikian and Breslauer, 1995; Chalikian *et al.*, 1995; Chalikian and Breslauer, 1996). Compressibility provides information about conformational transitions of proteins between these four states.

Chalikian *et al.* (1995) measured the partial specific adiabatic compressibility, $\bar{K}_S^o$, of cytochrome *c* to describe the four-state conformational transitions induced by acid as shown in Figure 2.3.
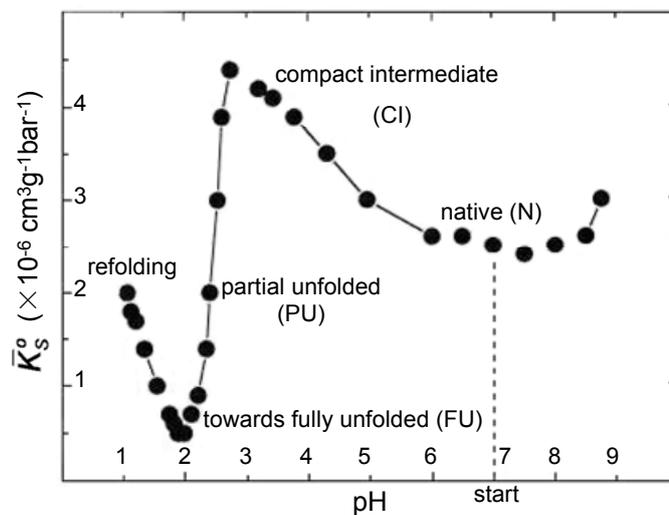
Figure 2.3 pH-dependence of the partial specific adiabatic compressibility, $\bar{K}_S^o$, of cytochrome *c* (Adapted from Journal of Molecular Biology, v. 250, TV Chalikian, VS Gindikin and KJ Breslauer, Volumetric characterizations of the native, molten globule and unfolded states of cytochrome c at acidic pH, *p.* 293, Copyright (1995), with permission from Elsevier).

The native state (N) has a unique tertiary structure with amino acid residues packed tightly and plenty of hydrophobic residues buried in the solvent inaccessible interior core of the protein. The stable values of $\bar{K}_S^o$ indicate that cytochrome c does not change conformation from the native state from pH=8 to pH=6. In the compact intermediate (CI) state, or molten globule (MG) state, the rigid tertiary structure is lost but the secondary structure (e.g., α-helices) is preserved, resulting in a loose hydrophobic core of the protein, and the value of $\bar{K}_S^o$ increases by 2×10^{-6} cm^{3}g^{-1}bar^{-1} compared with the N state. The partial unfolded state (PU) loses more tertiary and secondary structure than the compact intermediate state (CI) and the looser hydrophobic cavity becomes accessible to solvent. Thus, the value of $\bar{K}_S^o$ decreases. The fully unfolded state (FU) is randomly coiled and all the protein molecular groups are solvent accessible and here the protein has the smallest value of $\bar{K}_S^o$. It should be noted in Figure 2.3 that an increase in $\bar{K}_S^o$ of cytochrome c occurs at about pH<2.0. This increase can be explained by refolding of the unfolded proteins. The addition of more acid increases both protons and anions in the solution. Since cytochrome c is maximally protonated around pH=2, protons have no more effects on the ionization of proteins. However, more anions lead to neutralization of the positive charge on proteins, so the intra-molecular electrostatic repulsive forces are decreased. As a result, the protein molecule does not unfold fully and begins to refold (Goto *et al*., 1990；Chalikian *et al*., 1995).

There is a general relationship between conformational transitions of proteins

and the changes in their partial specific adiabatic compressibility. For most globular proteins, changes in partial specific adiabatic compressibility, $\Delta \bar{K}_S^o$, are estimated to be $+(1 \sim 4) \times 10^{-6}$ cm^{3}g^{-1}bar^{-1} for N to CI; $-(3 \sim 7) \times 10^{-6}$ cm^{3}g^{-1}bar^{-1} for N to PU; and $-(18 \sim 20) \times 10^{-6}$ cm^{3}g^{-1}bar^{-1} for N to FU transitions. That allows one to determine the types of protein transitions by changes in compressibility (Kharakoz and Sarvazyan, 1993; Chalikian *et al.*, 1995; Chalikian and Breslauer, 1996; Taulier and Chalikian, 2002; Dubins *et al.*, 2003).

Since protein compressibility is a sum of contributions of compressibility of the intrinsic core and the hydration shells, different conformational states are related to the intrinsic and hydration properties of a protein. The native states are the most compact conformation among these four possible states (N, CI, PU and FU). This compactness leads to a minimum value for the total solvent accessible surface area of proteins, $\theta_{acc} = \theta_{cha} + \theta_{pol} + \theta_{non}$. Thus, native proteins have smaller hydration area on the surfaces and the hydration shells have less negative contributions to the protein compressibility (less negative values of $\Delta \bar{K}_i^o$) than the proteins at the other three molecular states (CI, PU, and FU). Moreover, native proteins have the most tightly packing interior cores and the largest volumes of intrinsic cores, $\bar{v}_M^o$ (the volume confined by the molecular surface in Figure 2.2), among the four states (Chalikian *et al.*, 1995). Some of the buried residues are exposed to the solvent during the transition of a protein from native (N) to compact intermediate (CI) states and a smaller intrinsic core is preserved. Thus, the volume of the solvent inaccessible interior core (volume of the blank area inside the molecular surface in Figure 2.2) decreases, and the total solvent

accessible surface area, θ_{acc}, increases. As a result, there are more negative contributions to the protein compressibility from smaller solvent inaccessible volume inside the protein and larger hydration area on the protein surface. For instance, cytochrome *c* at CI states loses 60% of the intrinsic volume (essentially the void volume inside the molecule as represented by the blank area inside the molecular surface in Figure 2.2) and has roughly 60% more total solvent accessible surface area compared with that at N states (pH≈7) (Goto *et al.*, 1990). Therefore, one can expect a negative change in the partial specific adiabatic compressibility of the protein, $\overline{K}_S^o$, during the transition from the N state to the CI state. However, as seen in Figure 2.3, the value of $\overline{K}_S^o$, of a CI molecule is greater than the N molecule. That is explained by an increased partial specific intrinsic adiabatic compressibility coefficient, $\overline{\beta}_{SM}^o$, caused by loosening of the interior packing (Chalikian *et al.*, 1995; Taulier and Chalikian, 2002; Chalikian, 2003). Although the solvent inaccessible void space inside the protein molecule is lost during the state transition, the protein interior that is preserved (mainly the space between the black balls and not shown in Figure 2.2) is more compressible due to looser packing of the constituent atoms (black balls in Figure 2.2). Kharakoz and Sarvazyan (1993) proposed that the compressible behaviour of a native protein is comparable to that of organic solids. However, proteins in compact intermediate states have liquid-like compressible behaviour (Chalikian *et al.*, 1995). The acid-induced CI cytochrome *c* molecule has a partial specific intrinsic adiabatic compressibility coefficient, $\overline{\beta}_{SM}^o$ increase of 4.5 fold. This looser interior core of a

protein leads to a great positive contribution to $\bar{K}_S^o$ and overcomes the negative contributions from hydration shells. Thus, a net increase in partial specific adiabatic compressibility, $\bar{K}_S^o$, of the CI protein molecules is observed. As the transition continues, from the CI states to PU states, about 70% of the fully unfolded surface is exposed to the solvent, causing a much larger negative hydrational contribution (Lee, 1991; Chalikian *et al.*, 1995). The intrinsic core of a molecule at this stage is small but still preserved and more hydrophobic. In a similar situation to that occurring in the CI state, the interior packing of a protein is becoming looser, and the partial specific intrinsic adiabatic compressibility coefficient, $\bar{\beta}_{SM}^o$, is higher due to looser interior packing. However, the negative hydration contribution prevails over the positive intrinsic contribution as found in the acid-induced N-to-PU transition of cytochrome *c* by Chalikian *et al.* (1995) and the net decrease in partial specific adiabatic compressibility, $\bar{K}_S^o$, for this type of transition is observed. As for the N-to-FU transition, the protein molecule loses the solvent inaccessible core completely, so the protein is highly incompressible due to the large negative hydration contributions and the loss of its compressible interior structures, consistent with the large decrease in partial specific adiabatic compressibility, $\bar{K}_S^o$.

2.1.3.4. Functionalities and Compressibility Properties of Proteins

Gekko and Yamagami (1991) studied the protein partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, and the digestion rate at which enzymes break

the peptide bonds and found that enzymes break down more compressible (larger $\bar{\beta}_S^o$) proteins at higher digestion rates. Based on the assumption that flexible proteins are easier to be digested by enzymes, the compressible property, $\bar{\beta}_S^o$, of a protein is considered to indicate and provide a quantitative estimate of its molecular flexibility.

According to Graham and Phillips (1979a; 1979b; 1979c), molecular flexibility of a protein is an important characteristic related to its interface adsorption, which largely determines foaming and emulsifying behaviour. Protein interface adsorption involves three successive steps: (1) diffusion from the solution to the interface; (2) overcoming the energy barrier to adsorb at the interface; and (3) adsorption and conformational rearrangement at the interface (Le Meste *et al*., 1990). With a lower energy barrier in step (2) and an easier structural rearrangement in step (3), molecular flexibility improves the interface adsorption of proteins (Kato *et al*., 1985). Thus, the measurement of partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, of a protein provides a method to detect its interface functionalities (Gekko and Noguchi, 1979; Gekko and Hasegawa, 1986). Moreover, active interface behaviour of a protein can be predicted from its large value of partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$.

The foaming capacities and the partial specific adiabatic compressibility coefficients, $\bar{\beta}_S^o$, of different proteins were measured (Townsend and Nakai, 1983; Gekko and Yamagami, 1991) and the plot in Figure 2.4 gives the relationship. Compressible proteins exhibit strong foaming capacity. Figure 2.4 also shows a

critical value of $\bar{\beta}_S^o$ around 4×10^{-6} bar^{-1} for high foaming capacity. Because the

partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, of a protein is related to

its molecular flexibility and hydrophobicity, it can be interpreted that compressible

proteins (with $\bar{\beta}_S^o > 4 \times 10^{-6}$ bar^{-1}) allow non-polar groups to expose to the solvent

via easier rotations of the flexible backbones of their polypeptide chains,

increasing the hydrophobic interactions of protein with air and favouring protein

adsorption at the water-air interface.
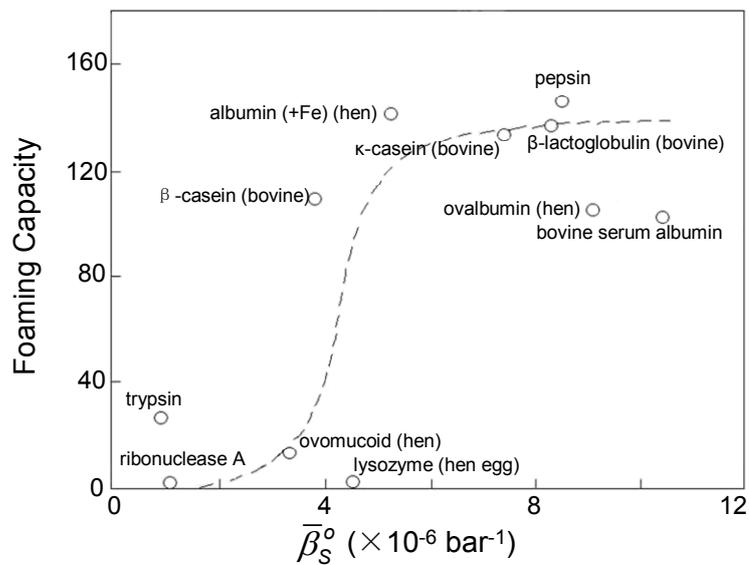
Figure 2.4 Foaming capacity versus the coefficient of partial specific adiabatic
compressibility coefficient, $\bar{\beta}_S^o$, of proteins (Adapted from Journal of
Agricultural and Food Chemistry, v. 39, K Gekko *et al.*, Flexibility of
food proteins as revealed by compressibility, *p*. 60, Copyright (1991),
with permission from American Chemical Society).

The free energy of protein unfolding in water, ΔG_{unf}, versus partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, is plotted in Figure 2.5 (Gekko and Yamagami, 1991). Figure 2.5 demonstrates that the compressible proteins ($\bar{\beta}_S^o > 4 \times 10^{-6}$ bar^{-1}) are generally easier to denature (unfold) in water than the rigid ones ($\bar{\beta}_S^o < 4 \times 10^{-6}$ bar^{-1}), suggesting a lower energy barrier for compressible proteins to perform good foaming and emulsifying behaviour.
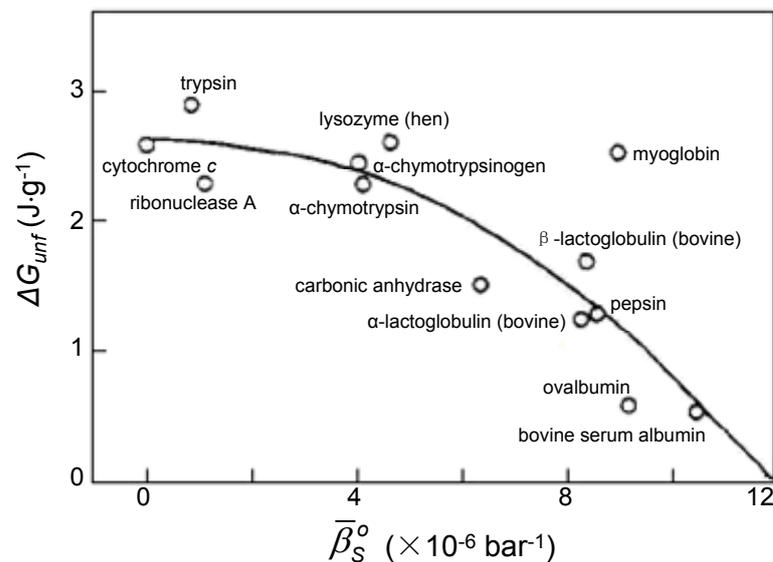
Figure 2.5 Plots of the free energy, ΔG_{unf}, of protein unfolding in water at 25 °C and pH=7, versus the partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ (Adapted from Journal of Agricultural and Food Chemistry, v. 39, K Gekko *et al.*, Flexibility of food proteins as revealed by compressibility, *p.* 61, Copyright (1991), with permission from American Chemical Society).

2.2. Introduction of Gliadins

2.2.1. Classification and Structures of Gliadins

Gliadins, as defined by Wieser (1996), are those protein components of wheat flours or wheat glutens that are insoluble in water or neutral salt solutions, but soluble in aqueous alcohols without reduction of disulphide bonds. Gliadin is not a simple protein but a complex mixture of numerous protein components.

On the basis of the mobilities in low pH gel electrophoresis, the gliadins were initially classified into four groups: α-, β-, γ-, and ω-gliadins, which were in the order of decreasing mobility (Woychik *et al.*, 1961; Wieser, 1996; Wieser, 2007). According to the analysis of primary structure and molecular weights (MWs), a new classification is given as ω5-, ω1,2-, α/β- and γ-gliadins (Wieser, 1996; Wieser, 2007). From this classification, α- and β-gliadins fall into one group because of small structural differences. The α/β-gliadins have overlapping MWs (≈ 28,000 ~ 35,000) with the γ-gliadins. The ω1,2-gliadins have MWs around 40,000 and the ω5-gliadins have higher MWs (≈ 50,000).

Wieser (1996) reviewed the characteristics of gliadin types as shown in Table 2.3. The ω-gliadins have high contents of glutamine, proline and phenylalanine and lack cysteine compared with α/β- and γ-gliadins. The distribution of different gliadin types depends on the genotype of wheat cultivars and growing conditions (such as soil, climate, fertilization, etc.). However, Wieser and Kieffer (2001) pointed out that α/β- and γ-gliadins are major components, accounting for 28-33% and 23-31%, respectively, but the ω-gliadins occur in much lower proportions,

only 4-7% and 3-6% for the ω1,2- and ω5-gliadins, respectively. Since cysteines form the intra-chain disulphide bonds in the gliadins, there should be an even number of these amino acid residues in one gliadin protein. However, there is a small amount of gliadins having an odd number of cysteines which is caused by point mutations. That makes the gliadins link together or to glutenins, appearing either in the alcohol-soluble gliadin fractions as oligomers with relative low molecular weights or in the alcohol-insoluble glutenin fractions as polymers with high molecular weights. The former oligomeric fraction containing α/β- and γ-gliadins and low-molecular-weight (LMW) glutenins linked by inter-chain disulphide bonds has been called high-molecular-weight (HMW) gliadin, aggregated gliadin or ethanol-soluble glutenin with the range of MWs around 100,000–500,000 (Shewry *et al*., 1983; Huebner and Bietz, 1993; Wieser, 2007).

Table 2.3 Characteristics of different gliadins (Wieser, 1996).

Amino acid	Composition (mol %)			
	ω5-	ω1,2-	α/β-	γ-
Gln	55-56	43-45	36-41	33-38
Pro	19-20	25-21	15-19	16-18
Phe	9-10	7-8	3-4	4-6
Tyr	0-1	1-2	3-4	0-1
Cys	0	0	1-2	2-3

Tatham and Shewry (1985) studied the secondary structures of gliadins using circular dichroism spectroscopy and found that ω-gliadins were rich in randomly coiled β-turns without detectable α-helix or β-sheet, but α/β- and γ-gliadins contained 30-35% α-helix and 10-20% β-sheet conformations. They also reported that ω-gliadins were mainly stabilized by strong hydrophobic interactions and α/β- and γ-gliadins were stabilized by covalent disulphide bonds and non-covalent hydrogen bonds in their α-helices and β-sheets.

Given that there are different types of gliadins and various secondary structure associated with these molecules, there is no consensus on the structure of gliadins in solutions. For example, Friedli (1996) proposed a doughnut-like structure for gliadin molecules in which there was a large central hole. In contrast, it has been reported that the α/β-gliadins have compact globular structures and γ- and ω-gliadins have extended and rod-like structures (Paananen *et al.*, 2006; Ang *et al.*, 2010). However, gliadins are still widely considered to adopt a globular protein structure in 70% aqueous ethanol (Gennadios and Weller, 1990; Foulk and Bunn, 2001).

2.2.2. Extraction of Gliadins

The Osborne fractionation scheme of wheat proteins might be the most famous and important landmark in the history of cereal science. In Osborne's scheme, wheat proteins were grouped into four classes according to their different solubilities in sequential extraction solvents. The albumins were considered as the soluble fraction in water; the globulins were soluble in salt solutions; the gliadins

were soluble in 70% to 90% (v/v) aqueous ethanol; and the glutenins were soluble in dilute acid or alkali (Osborne, 1907). According to this classification, gliadins can be obtained by sequential extractions using water, salt solutions and finally aqueous ethanol solutions. Chen and Bushuk (1970) modified Osborne's method by starting with dilute (0.5 M) NaCl not water. However, the classical and modified Osborne sequential fractionation is not effective enough to obtain clean fractions because of the high heterogeneity of wheat proteins and the strong non-covalent interactions between gliadin and glutenin proteins caused by salts.

Fractionation and purification of wheat flour proteins has been a popular topic to cereal chemists for a long time and a lot of fractionation and purification methods of wheat flour proteins have been developed. However, there are not many reports on extractions of gliadins directly from flours, and most of the researches were focused on the separation of glutenins from monomeric proteins (albumins, globulins and gliadins). In this section only the extraction methods of gliadins are covered because this study is only on crude gliadin proteins of wheat flours.

Fu and Sapirstein (1996) separated monomeric proteins (albumins, globulins and gliadins) from polymeric glutenins in Glenlea and Katepwa flours using 50% (v/v) and 70% (v/v) aqueous 1-propanol solutions. They extracted proteins with 50% (v/v) 1-propanol to obtain soluble and insoluble fractions. Then they took some of the 50% (v/v) 1-propanol soluble fractions and added 1-propanol to bring the final 1-propanol concentration to 70% (v/v). Thus, four fractions were obtained: 50% (v/v) 1-propanol soluble and insoluble fractions, and 70% (v/v) 1-propanol

soluble and insoluble fractions, which were the subfractions of the 50% (v/v) 1-propanol soluble fraction. In a subsequent protein analysis with A-PAGE, SDS-PAGE and RP-HPLC, the authors concluded that the 50% (v/v) 1-propanol soluble protein was mainly gliadin but also contained albumin and globulin and some lesser proportion of polymeric glutenins (LMW-glutenins and HMW-glutenins linked by intermolecular disulphide bonds) and the 70% (v/v) 1-propanol soluble protein contained only monomeric proteins (mainly gliadins). Their work showed that sequencial extraction from wheat flour with 70% (v/v) aqueous 1-propanol solution was one effective method to obtain monomeric proteins, most of which were gliadins.

Fu and Kovac (1999) extracted monomeric proteins (albumins, globulins and gliadins) from glutenins in Glenlea, Neepawa, and AC Karma flours using NaI – 1-propanol aqueous solutions. Various concentrated NaI (0.25 to 2.0 M, with 0.25 M increments) and 1-propanol [5–50% (v/v), with 5% increments] aqueous solutions and their combinations [NaI dissolved in different 1-propanol aqueous solutions] were used and they found that the extraction using 0.3 M NaI – 7.5% (v/v) 1-propanol aqueous solution was the most effective method to separate the two protein classes. Dupont *et al*. (2005) modified Fu and Kovac's fractionation procedure and separated gliadins from albumins and globulins. They extracted flours with the 0.3 M NaI – 7.5% 1-propanol aqueous solution and collected the supernatant fractions that were rich in monomeric proteins (albumins, globulins and gliadins). Then, four volumes of cold (-20 °C) NH_4Ac-MeOH were added to the supernatant fractions and stored at -20 °C for 48 h. The precipitates caused by

adding NH_4Ac-MeOH were collected by centrifugations. In subsequent RP-HPLC and SDS-PAGE analyses of proteins, they found that the precipitates were gliadins contaminated by a small amount of albumins and globulins as well as glutenins. The scheme of Dupont *et al*. might be the most effective method of extraction of gliadins from flours.

2.2.3. Crude Gliadins from Alcohol Extractions

The 70% (v/v) ethanolic extracts of flours or glutens are considered as classic gliadins (Hoseney *et al*., 1969b), and the composition of this fraction has been widely studied. Ponte *et al*. (1967) fractionated acid-soluble gluten into gliadins and glutenins with 70% ethanol and found that the gliadin fraction contained about 73.6% proteins and 9.1% lipid and there was no starch in it. Similar results were observed by Hoseney *et al*. (1969b) who obtained the 70% ethanolic soluble fraction of gluten of regional baking standard (RBS) flours and found that the extracts contained 9% lipid. Hoseney *et al*. (1969b) also proposed that 70% aqueous ethanol changed the interaction of lipids with gliadins and glutenins and caused more lipids to be soluble in the gliadin fraction. Békés *et al.* (1983a) fractionated glutens of differently defatted Neepawa flours and found the gluten from undefatted flour contained 8.95% of lipids. Furthermore, they also found that glutens with higher lipid content yield larger amount of ethanol soluble classic gliadins. Based on these observations, Békés *et al.* (1983a) presumed that some proteins formed complexes with lipids and were more soluble in 70% aqueous ethanol. Zawistowska *et al.* (1984) extracted classic gliadin fractions directly from

flours (Sinton, Neepwa, Era, Glenlea and Tesopaco) with 70% aqueous ethanol and found the lipids accounted for 11.5-13.5% of the gliadin fraction. In subsequent research they assumed that there were complexes existing formed by lipids and some specific proteins in the 70% ethanol soluble gliadin-rich fraction, which was consistent with the conclusions of Békés *et al.* (1983b) in the studies of Neepawa flour glutens.

It has been reported that the gliadin-rich fraction obtained with 70% aqueous ethanol contained no starch (Ponte *et al.*, 1967). However, there are some carbohydrates in the alcohol-soluble fraction. McMaster and Bushuk (1983) extracted gliadins from glutens with 70% aqueous ethanol and found that the ethanol soluble fraction contained 0.6% (w/w) carbohydrates. Besides, in a sequential gel chromatography experiment, they found most of the carbohydrates existed in the high molecular fractions (i.e., the glutenins) of the extracts. However, there was no evidence to show whether or not the carbohydrates and the proteins were linked to each other.

2.2.4. Functionalities of Gliadins

It is well known that the gluten proteins, including gliadins and glutenins, determine the dough mixing properties of flours and their suitability for breadmaking. Many studies have been performed on the functionality of gliadins in the process of dough mixing and bread making.

Hoseney *et al*. (1969a) used 0.005 M lactic acid (pH=4.7) to fractionate gluten proteins. The insoluble fraction was assumed to be glutenins and showed no

effect on the loaf volume potentials. The soluble fraction at pH 4.7 was then separated into two portions, the supernatant and the sediment, by ultracentrifugation at 100,000 g for 5 h, and the sediment was also taken as glutenins. After the ultracentrifugation they found that the supernatant portion, rich in gliadins, accounted for the change in loaf volume potentials. Based on the assumption that the glutenins behaved similarly in the insoluble and soluble fractions, the authors pointed out that the gliadins governed the loaf volume potential of wheat flours.

Preston and Tipples (1980) used 0.05 M acetic acid to separate gluten proteins of hard red spring wheat flours into two fractions, acid-soluble and acid-insoluble proteins. These two fractions were lyophilized and ground into powders. In successive studies, the powders of acid-soluble and acid-insoluble proteins were respectively added to different base flours and the mixing and baking properties of these flours were tested. The conclusion obtained from this study was that the acid-soluble gluten increased the loaf volumes of base flours while the acid-insoluble fraction caused lowering of loaf volumes. However, the functionality of gliadins in flour baking quality can not be confirmed in this study because the acid-soluble fraction accounted for 85% of the total gluten proteins, which means that the gliadins in the acid-soluble fraction were largely contaminated by glutenins.

MacRitchie (1987) successively extracted proteins from wheat flours using diluted HCl with different pH values. The extracts with diluted HCl of high pH values (about from 5.0 to 5.8) were taken as gliadin-rich proteins and when added

to the base flours increased the protein level by 1%. In subsequent tests, the fortified flours were assessed based on their mixograph peak development times and loaf volumes, and it was found that the gliadin-rich fractions decreased dough strength and slightly depressed loaf volumes.

The functionality of gliadins is difficult to determine because of the poor isolation and purification techniques for gliadins. The gliadins themselves are made up of many different protein components and the contamination from LMW-glutenins enhances the difficulties in studies on gliadins (Fido *et al.*, 1997; Khatkar *et al.*, 2002).

Khatkar *et al.* (2002) extracted gliadins from flours using 70% (v/v) aqueous ethanol and purified and separated the gliadin subgroups with carboxymethyl cellulose chromatography without protein aggregation. It had been reported that in these gliadin subgroups, the α- and ω1,2-gliadin fractions were essentially free from other gliadin components and the β-gliadins showed some minor contamination with γ-gliadins (Fido *et al.*, 1997). The total gliadins and purified gliadin subgroups were then added into the base flour and the mixing and breadmaking properties of the treated flours were studied. In their conclusion, it was stated that the gliadins tended to decrease the overall dough strength and stability and both the total gliadins and individual gliadin subgroup substantially increased the loaf volume and improved the pan bread making quality of the flours.

Békés *et al.* (1992) extracted gliadin-rich fractions with 70% aqueous ethanol from flours of 15 different wheat cultivars. Sequentially, they fractionated the

gliadin-rich extracts using size exclusion high performance liquid chromatography (SE-HPLC) and obtained the peak for lipid mediated aggregates (LMA). The authors pointed out that these aggregates were formed by alcohol soluble LMW-glutenins and lipids. Also, the authors found a statistical relationship between the amount of LMA and the loaf volume of the flours.

Different from other studies on gliadin functionalities, the work of Békés *et al*. took the protein-lipid interactions in gliadin-rich extracts into account and this might provide a better understanding of the properties of gliadin-rich extracts.

3. Materials and Methods

3.1. Materials

3.1.1. Wheat Cultivars

The preliminary aqueous alcohol analyses were performed using flours of four different wheat cultivars: Glenlea, Katepwa, AC Reed and Genesis.

The solvent analyses were performed using a single source of No. 1 Canada Western Red Spring 13.5 (1 CWRS 13.5) wheat flour which had been milled as a straight grade flour at the Canadian International Grains Institute (CIGI, Winnipeg, Manitoba) pilot mill. 1 CWRS 13.5 is a blend of hard wheat cultivars with excellent milling, mixing and baking qualities.

The subsequent flour analyses were performed using three flour sources with different grades: No. 1 Canada Western Extra Strong (1 CWES), a hard red spring wheat with extra-strong gluten suitable for blending purposes and for special breads, and No. 1 Soft White Spring (1 SWS), a soft wheat of low protein content.

3.1.2. Chemical and Reagents

The chemicals and reagents used in this study are listed in Table 3.1.

3.1.3. Instruments and Supplies

The instruments and supplies used in this study are listed in Table 3.2.

Table 3.1 Chemicals and Reagents.

Chemicals	Manufacturer
Ethanol (70% v/v)	Fisher Scientific Inc., Nepean, ON.
Boric acid	Mallinckrodt Canada Inc., Pointe-Claire, QC.
Copper sulphate	Pope Kjeldahl Mixtures Inc., USA.
Ethanol (95% v/v)	Commercial Alcohols Inc., Brampton, ON.
Ethylene glycol	Canadian Tire
Glacial acetic acid	Fisher Scientific Inc., Nepean, ON.
Hydrochloric acid	Fisher Scientific Inc., Nepean, ON.
Methyl blue	Sigma-Aldrich, St. Louis, MO.
Methyl red	Sigma-Aldrich, St. Louis, MO.
Potassium sulphate	Pope Kjeldahl Mixtures Inc., USA.
Sodium chloride	Fisher Scientific Inc., Nepean, ON.
Sodium hydroxide	Fisher Scientific Inc., Nepean, ON.
Sulphuric acid (98%)	Fisher Scientific Inc., Nepean, ON.
Titanium dioxide	Pope Kjeldahl Mixtures Inc., USA.

Table 3.2 Instruments and Supplies.

Instruments and Supplies	Manufacturer
0-1 mL, 0-5 mL pipettes	Fisher Scientific Inc., Nepean, ON.
250 µL syringe	Hamilton Company, Switzerland.
Centrifuge (RC5C)	Sorvall Instruments, USA.
DMA 5000 density meter	Anton Paar, Austria.
Pilot lyophilizer	VirTis, USA.
Kjeldahl system 1002 distilling unit	FOSS Tecator AB, Sweden.
Micro-centrifuge (Biofuge A c1710-20)	Heraeus-Christ GmbH, Germany.
Oil bath (Isotemp 3028)	Thermo Fisher Scientific, USA.
ResoScan System	TF Instruments Inc., Germany.
VC 60 ultrasonic processor	Sonics & Materials, Inc., USA.

3.2. Methods

3.2.1. Extraction of Crude Gliadins

3.2.1.1. Extraction of Gliadins Using an Osborne Fractionation

For the preliminary aqueous alcohol analyses, crude gliadins were extracted following the modified Osborne fractionation (Chen and Bushuk, 1970). Flours (250 mg) were extracted twice at room temperature (23 ± 1 °C) with 1 mL of 0.5 M NaCl solution in a micro-centrifuge tube for 10 min with brief vortexing every 5 min. Each suspension was centrifuged for 10 min at 3180 g, and the supernatant was decanted. The residue was washed with 1 mL of distilled deionised water for 5 min for 2 times, followed by centrifugation. The residue was then extracted with 1 mL 70% (v/v) aqueous alcohol for 10 min at room temperature, with brief vortexing every 5 min. The suspension was then centrifuged for 10 min at 3180 g. The procedure was repeated 3 times and the 4 supernatants of alcohol extractions of each flour sample were poured into a test tube and combined. Then, 1 mL of the combined extraction was transferred to a micro-centrifuge tube whose mass had been determined. This 1 mL of solution was dried with nitrogen gas and the micro-centrifuge tubes with the residues inside were weighed. Then the rough amount of extracted crude gliadins could be known. The rest of the extraction was used for making serial dilutions for ultrasonic velocity measurements.

3.2.1.2. Extraction of Crude Gliadins Using Aqueous Ethanol Solution

Crude gliadins extracted with 70% (v/v) aqueous ethanol solution were used for the solvent and the flour analyses. The extractions were selected to exclude the influence from the residual salt in the Osborne fractionation (Arrhenius, 1937). Wheat flour samples (25 g) were mixed with 70% (v/v) aqueous ethanol solutions (100 mL) at room temperature (23 ± 1 °C). The mixture was shaken for 30 min and then was centrifuged at 478 g for 10 min. The supernatant was poured into a container and dried with air. The residue was then lyophilized in a freeze drier. Five extractions were performed to prepare sufficient freeze-dried materials. The products from the five extractions were mixed and ground into powders. The powders were used in the study as crude gliadins and stored at 4 °C. The predominant proteins in these extracts are considered as gliadins according to Robertson *et al*. (2007).

3.2.1.3. Extraction of Gliadins at Low Temperature

Crude gliadins were extracted with 70% (v/v) aqueous ethanol at low temperatures (Arrhenius, 1937) to evaluate the efficacy of the extraction method in *Section 3.2.1.2* for obtaining pure gliadins. Wheat flour samples (25 g) were extracted with 70% (v/v) aqueous ethanol solutions (100 mL) at room temperature (23 ± 1 °C). The mixture was stirred for one-half day and then was put into an oil-bath at -8 °C for 4 days. The mixture was centrifuged at 478 g for 10 min at 4 °C and the supernatant was poured into a container and aspirated with air to let

the ethanol evaporate. When most of the alcohol had been removed (almost no alcoholic smell) the supernatant containing both water soluble materials and some gliadins was carefully discarded. The residue was then lyophilized in a freeze drier. The products from the lyophilization were ground into powders. The protein content of these powders were determined by Kjeldahl method and compared with the results of *Section 3.2.1.2*.

3.2.2. Preparation of Crude Gliadin Solutions

The proteins dissolved in liquid from *3.2.1.1* and the solid gliadins from *3.2.1.2* were prepared for testing by performing a series of serial dilutions.

3.2.2.1. Preliminary Aqueous Alcohol Analyses

The serial dilutions were made up using the extraction sealed in the test tube. Approximately 3 mL of the extractions from *Section 3.2.1.1* was the parent solution for making the serial dilutions with a 1 mL pipette as shown in Figure 3.1. The 70% (v/v) alcohol was used as the solvent. According to the procedure shown in Figure 3.1, the parent solution was serially diluted with 70% (v/v) alcohol and the dilution rates of 1, 1/2, 1/5, 1/10, 1/20, 1/50, and 1/100 were obtained. The final volumes of the serial dilutions are listed in Table 3.3.
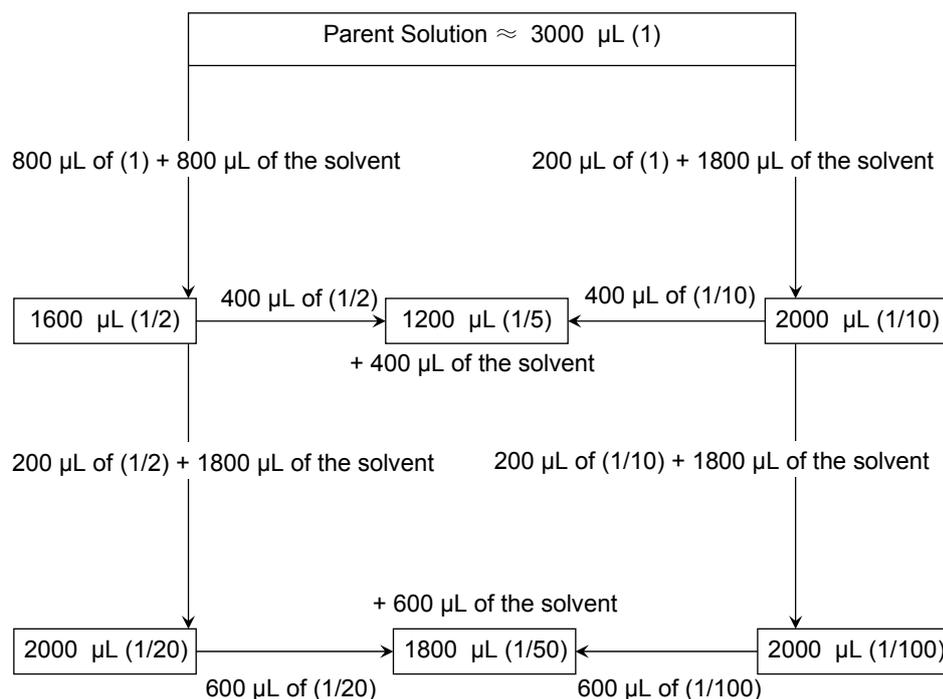
Figure 3.1 The procedure of making up serial dilutions for preliminary aqueous alcohol analyses. Numbers in brackets indicate the dilution.

3.2.2.2. Solvent and Flour Analyses

For the solvent and the flour analyses, about 10 mL of each serial dilution of crude gliadin solutions was made up for the density and ultrasound velocity measurements. Extract concentrations of dissolved crude gliadins were determined following procedures of *Section 3.2.5*. Two solvents (70% v/v aqueous ethanol or 4 mM acetic acid) were used to dissolve the crude gliadins and to make up the dilutions in the solvent analyses, but only the 4 mM acetic acid was used for the subsequent flour analyses. For the solvent analyses, 375.0 ± 0.1 mg of crude gliadin powder was weighed and mixed with 30 mL of solvent which was

measured with a 50 mL volumetric cylinder. The dissolving procedure was a 10 min dissolution with brief vortexing every 3 min at room temperature (23 ± 1 °C). The suspensions were centrifuged at 17200 g for 10 min after either dissolving procedure. The supernatant (≈ 30 mL) was poured into a covered test tube and sealed. From the 30 mL of supernatant of the parent crude gliadins in either ethanol or acetic acid, 10 mL of the solution was retained as the most concentrated solution, 10 mL of the rest was used for 1/2, 1/4, 1/8 and 1/16 serial dilutions with a 5 mL pipette. The rest of the supernatant was used for micro-Kjeldahl nitrogen determination methods. The detailed procedure of serial dilutions for the solvent analysis is shown in Figure 3.2.

For the subsequent flour analyses, the same dissolving procedure using 4 mM acetic acid was performed except that two supernatants after centrifugation (≈ 30 mL for each) were poured into an 80 mL beaker, mixed and sealed with parafilm. From the 60 mL of supernatant of the parent crude gliadins in acetic acid, 10 mL of the solution was retained as the most concentrated solution, 15 mL and 10 mL of the rest were used for 3/4, 3/8, and 1/2, 1/4 serial dilutions, respectively. The same 5 mL pipette as the solvent analysis was used. The rest of the supernatant was used for micro-Kjeldahl nitrogen determination methods. The detailed procedure of serial dilutions for the flour analysis is shown in Figure 3.3.

```
                    ┌──────────────────────────────────┐
                    │ Parent Solution ≈ 30 mL (1)      │
                    └──────────────────────────────────┘
                                   │
         10 mL of (1) + 10 mL of the solvent
                                   ↓
                    ┌──────────────────────────────────┐
                    │          20 mL (1/2)             │
                    └──────────────────────────────────┘
                                   │
         10 mL of (1/2) + 10 mL of the solvent
                                   ↓
                    ┌──────────────────────────────────┐
                    │          20 mL (1/4)             │
                    └──────────────────────────────────┘
                                   │
         10 mL of (1/4) + 10 mL of the solvent
                                   ↓
                    ┌──────────────────────────────────┐
                    │          20 mL (1/8)             │
                    └──────────────────────────────────┘
                                   │
         10 mL of (1/8) + 10 mL of the solvent
                                   ↓
                    ┌──────────────────────────────────┐
                    │          20 mL (1/16)            │
                    └──────────────────────────────────┘
```
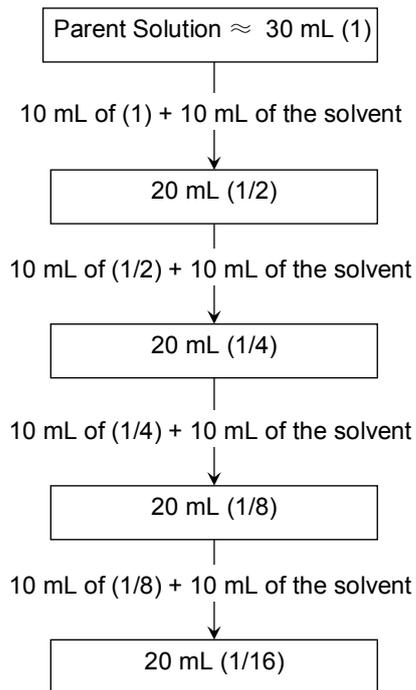
Figure 3.2 The procedure of making up serial dilutions for solvent analyses. Numbers in brackets indicate the dilution.

```
                    ┌──────────────────────────────────┐
                    │ Parent Solution ≈ 60 mL (1)      │
                    └──────────────────────────────────┘
                         │                    │
    15 mL of (1) + 5 mL of the solvent        │
                         ↓         10 mL of (1) + 10 mL of the solvent
    ┌──────────────────────┐                  ↓
    │    20 mL (3/4)       │         ┌──────────────────────┐
    └──────────────────────┘         │    20 mL (1/2)       │
                         │           └──────────────────────┘
  10 mL of (3/4) + 10 mL of the solvent       │
                         ↓         10 mL of (1/2) + 10 mL of the solvent
    ┌──────────────────────┐                  │
    │    20 mL (3/8)       │                  ↓
    └──────────────────────┘         ┌──────────────────────┐
                                     │    20 mL (1/4)       │
                                     └──────────────────────┘
```
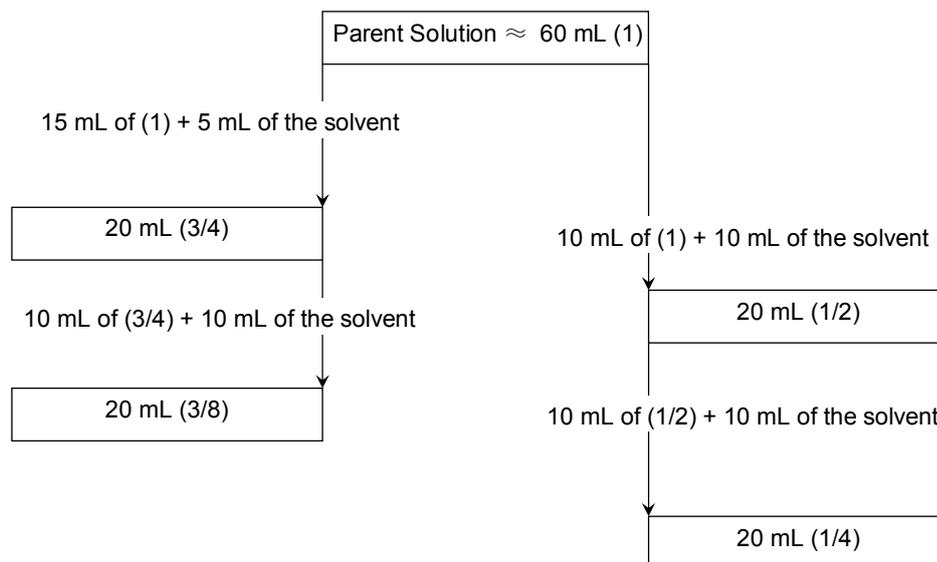
Figure 3.3 The procedure of making up serial dilutions for flour analyses. Numbers in brackets indicate the dilution.

3.2.2.3. Ultrasonication in Solvent Analyses

Preliminary experiments had shown that the crude gliadin powders did not dissolve in the 70% (v/v) aqueous ethanol completely. This was thought to be caused by aggregation of protein molecules during the freeze drying procedure. Thus, ultrasonication was used as an experimental treatment with a view to increasing the solubility of the crude gliadins.

A VC 60 ultrasonic processor (Sonics & Materials, Inc., USA) was used to sonicate the parent solution of the serial dilutions. In the preliminary experiments, 30 W for 3 min and 60 W for 1.5 min of sonication were applied to the 70% (v/v) aqueous ethanol and 4 mM acetic acid crude gliadin solutions. The suspensions were centrifuged at 17200 g and the supernatants were poured into the test tubes and applied to micro-Kjeldahl nitrogen determinations. The Kjeldahl results had shown that both sonication treatments had a positive effect on the solubility of the crude gliadins but neither of them made the powders completely soluble in different solvents. Additionally, there was no difference in the protein solubility values observed between the solutions after 30 W × 3 min and 60 W × 1.5 min sonication treatments. The increase in temperature of both solutions was kept below 7 °C during the sonication treatments when vials were inserted in cold water for cooling and the temperature rise was considered to have little influence on the evaporation of the solvents.

For the solvent analyses with two solvents (70% v/v aqueous ethanol or 4 mM acetic acid), sonicated solutions and non-sinicated ones were used in the determination of compressibility. The dissolving procedure of the ultrasonicated

solutions was the same as the non-sonicated ones except that an ultrasonication treatment was applied just before the last vortexing. Since there was no difference determined in the protein content of the gliadin solutions after 30 W × 3 min and 60 W × 1.5 min sonication treatments, only the 60 W × 1.5 min sonication treatment was selected. The subsequent serial dilutions procedure was exactly the same as the non-sonicated ones and is shown in Figure 3.2.

3.2.3. Ultrasound Velocity Measurements

The velocity of the ultrasonic wave passing through each of the liquid samples was determined with a ResoScan System (TF Instrument Inc., Germany). The ResoScan System comprises a special metal block thermostat for sample temperature control with a precision of about ± 0.003 K. According to the temperature dependence of the sound velocity in water at room temperature, which is 3 m·s^{-1} per K (Del Grosso and Mader, 1972), the ResoScan System can provide a measurement of ultrasound velocity in water with a precision as high as ± 0.01 m·s^{-1}.

The ultrasound velocity measurements were applied to the samples for all the analyses. Initially, the two measuring cells of the instrument were filled with distilled water before each measurement so that a cleanliness check was performed. The ultrasound velocities in the two cells were measured and the difference between them was calculated by the instrument. If the difference in velocity for the water in the two cells was smaller than 0.05 m·s^{-1}, the two cells

were considered clean enough so that the measurements could be continued. Otherwise, the two cells were rinsed with pure water and the cleanliness check was performed again.

The measurements began with the solvents and were continued from the most dilute solutions to the less dilute ones. For the preliminary aqueous alcohol analyses and the solvent analyses, measuring cell 1 was loaded with water and cell 2 was loaded with the sample. A 250 µL syringe (Hamilton Company, Switzerland) was used for sample loadings. The syringe was rinsed twice with precisely 200 µL of the solution. Measuring cell 2 was then rinsed twice with 200 µL of the solution. After rinsing, 200 µL of the solution was sucked precisely with the syringe and injected into cell 2. Cell 2 then was covered with a cap and the measurement was performed after the temperature in the cells was stable (20 ± 0.02 °C). Each measurement was ended after 10 readings (≈ 100 s). Then the rinsing and the measuring procedures were repeated for the next solution.

After all the measurements for each experiment, cell 2 was washed with water and with two special washing agents to remove the proteins from the measuring cell. Then both cells were washed with distilled water and loaded with distilled water to check the cleanliness of the cells. The instrument was turned off when the difference in velocity between the water in the two cells was not larger than 0.05 m·s^{-1}.

3.2.4. Density Measurements

Density measurements were performed on the samples for the solvent and the flour analyses. The densities of each of the serial dilutions were determined with a DMA 5000 density meter (Anton Paar, Austria) in the Canadian Malting Barley Technical Centre (CMBTC, Winnipeg, Manitoba). The instrument provides fast, easy and accurate determination of liquid density, with an accuracy of 5×10^{-6} $g \cdot cm^{-3}$ and a measuring range of 0 to 3 $g \cdot cm^{-3}$. The DMA 5000 density meter uses a high-precision Platinum thermometer to control and measure the temperature with a precision of $\pm$ 0.01 K. The viscosity of the liquid samples could destroy the accuracy of the density measurements and the limitation of the sample's viscosity is 700 mPas for the DMA 5000 density meter.

The density measurements began with the solvents and were continued from the most dilute solutions to the less dilute ones. For each measurement, about 3.5 mL of solution was aspirated into the measuring tube automatically and after the temperature was stable (20 $\pm$ 0.006 °C) the density of the solution was determined. After each measurement the measuring tube was automatically washed with water once and with methanol twice. At the end of the second wash with methanol, the measuring tube was blow-dried with air and the next solution was measured. The whole procedure took 10 -12 min.

3.2.5. Concentration of Crude Gliadin Solutions

Micro-Kjeldahl nitrogen determination was applied to the samples for the solvent and the flour analyses. For the solvent analyses, from the approximately 10 mL of solution, 1.5 mL, 2.0 mL and 2.5 mL of the supernatant were transferred to three digestion tubes with a 5 mL pipette. For the flour analyses, 1.0 mL, 2.0 mL and 3.0 mL of the supernatant were transferred to three digestion tubes with a 1 mL pipette. The tubes were dried using a digestion oven at 120 °C for about 2 hours. After the liquids in the tubes evaporated completely, the tubes were cooled for about 20 min to reach room temperature. One scoop ($\approx$1.234g) of pre-mixed catalyst (containing 10g K_2SO_4, 0.3g TiO_2 and 0.2g $Cu\ SO_4$) was added into the dried tubes and 3 mL of 98% sulphuric acid was added to each tube. The samples were digested at 350 °C for 1 h and then cooled for 20 min to reach room temperature. The cooled liquids were added to 10 mL of water and boiled in a Kjeldahl system 1002 distilling unit (FOSS Tecator AB, Sweden). An excess of 50% (w/v) aqueous sodium hydroxide solution was added to the digestion tubes and the ammonia in water was collected using a 120 mL conical beaker with 10 mL of purple indicator (2 L of the aqueous solution containing 80 g boric acid, 14.3 mL of 0.2% of methyl red and 5.7 mL of 0.1% of methyl blue in ethanol) in it. As the aqueous ammonia flowed into the conical beaker, the color of the liquid changed from purple to green, indicating there was aqueous ammonia collected in the conical beaker. After the volume of the green liquid reached 50 mL, the distillation procedure was stopped and the green liquid in the conical beaker was titrated using 0.03004 N aqueous hydrochloric acid. The volume of the HCl used

for titration was recorded and the nitrogen content of the solution was calculated. The protein content was calculated by: [protein] = (mL of HCl titrated for samples – mL of HCl titrated for blank) × normality of HCl × equivalent weight of Nitrogen × 5.7 / (mL of sample volume), where normality of HCl was 0.03004 N and equivalent weight of Nitrogen is 14. The regular conversion factor from nitrogen to protein for wheat products is 5.7 (American Association of Cereal Chemists, 2000).

In order to obtain the concentration of crude gliadins in the solution from the protein concentrations, the parent solutions for the solvent analyses were made up following the procedures in *section 3.2.2.2* and *3.2.2.3*. The centrifuged supernatants were divided into two aliquots, from which about 6 mL was used for Micro-Kjeldahl nitrogen determination following the procedures above and the rest was transferred to two containers, 10 mL for each container, with a 5 mL pipette. The masses of the containers had been determined. The two 10 mL of solutions in the containers were aspirated overnight with air and freeze dried. The residues with the containers were weighed to determine total soluble solids content. The micro-Kjeldahl and dry-weight results gave the soluble protein and the soluble crude gliadin concentrations of the solution, so that a conversion value for soluble protein to soluble crude gliadins could be made.

3.3. Experimental Design

Experimental designs of preliminary, solvent and flour analyses are shown in Figure 3.4, Figure 3.5 and Figure 3.6, respectively. For the preliminary analyses, only the determinations of ultrasound velocity were measured on each of 7 serial dilutions of each extraction of four wheat flours. For the solvent analyses, determinations of density and ultrasound velocity were measured on each of 12 solutions. This encompassed the two solvents (4 mM acetic acid and 62.65% w/w aqueous ethanol), and five serial dilutions of each solvent. As indicated in *section 3.2.2.3*, sonication or without sonication dissolutions were applied and triplicates of each treatment were performed. In order to randomize the experiment, the order of sonication of samples made with the same solvent was decided based on flipping coins. For the flour analyses, density and ultrasound velocity were measured on each of the serial dilutions of each extraction from three wheat flours and triplicates of each extraction were performed.
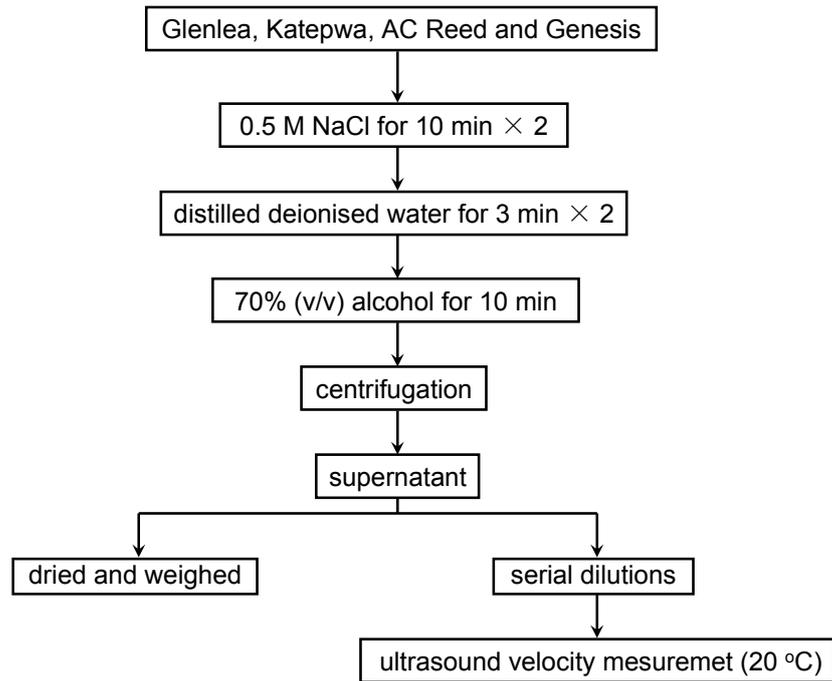
Figure 3.4 Experimental design for the preliminary analyses.
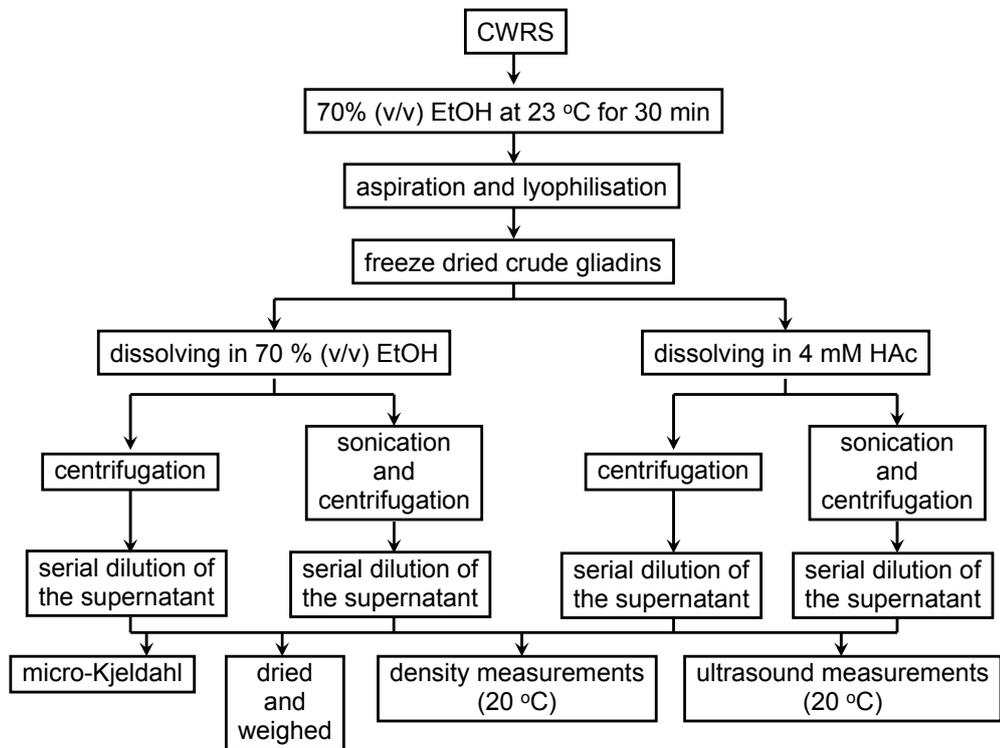
Figure 3.5 Experimental design for the solvent analyses.

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                    ┌──────────────────────┐
                    │    CWES and SWS      │
                    └──────────┬───────────┘
                               ▼
            ┌──────────────────────────────────────┐
            │  70% (v/v) EtOH at 23 °C for 30 min  │
            └──────────────────┬───────────────────┘
                               ▼
              ┌──────────────────────────────────┐
              │   aspiration and lyophilisation  │
              └────────────────┬─────────────────┘
                               ▼
               ┌─────────────────────────────────┐
               │   freeze dried crude gliadins   │
               └───────────────┬─────────────────┘
                               ▼
                 ┌────────────────────────────┐
                 │   dissolving in 4 mM HAc   │
                 └─────────────┬──────────────┘
                               ▼
                     ┌──────────────────┐
                     │   centrifugation │
                     └────────┬─────────┘
                              ▼
              ┌────────────────────────────────────┐
              │  serial dilution of the supernatant│
              └──────┬────────┬─────────┬──────────┘
         ┌───────────┘        │         │          └───────────┐
         ▼            ▼        ▼                    ▼
  ┌──────────────┐ ┌────────┐ ┌──────────────────┐ ┌────────────────────┐
  │micro-Kjeldahl│ │ dried  │ │density measurements││ultrasound measurements│
  └──────────────┘ │  and   │ │     (20 °C)      │ │       (20 °C)      │
                   │weighed │ └──────────────────┘ └────────────────────┘
                   └────────┘
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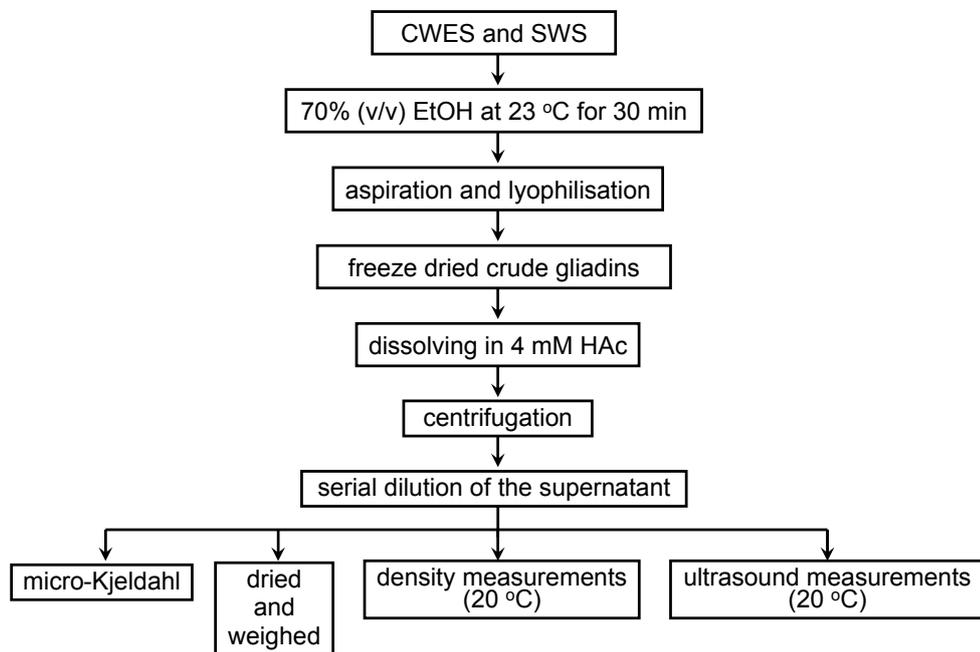
Figure 3.6 Experimental design for the flour analyses.

4. Preliminary Analyses Results and Discussion

The first stage of this project involved an evaluation of the extraction method of the crude gliadins from wheat flours. Only those solvents and gliadin solutions that display good repeatability in ultrasound velocity measurements can be used for experiments, so the preliminary analyses are necessary.

4.1. Preliminary Analyses on Osborne Gliadin-Rich Fractions

4.1.1. Content of Crude Gliadins in Extractions

Four varieties of wheat flours were used for the preliminary analyses (*Section 3.1.1*). Table 4.1 shows the concentrations of crude gliadins of two replicates with the modified Osborne fractionation method (Chen and Bushuk, 1970).

Table 4.1 Concentrations of crude gliadins (as solute weight) in the ethanolic extractions from modified Osborne fractionation.

Source of crude gliadins	Replicate 1 ($mg·mL^{-1}$)	Replicate 2 ($mg·mL^{-1}$)
Glenlea	1.9	3.6
Katepwa	2.3	3.9
AC Reed	2.9	4.7
Genesis	1.8	3.9

Results show poor repeatability in the amount of crude gliadins extracted from the same wheat flour between two replicates but consistency among four extractions within the same replicate. That is probably because of the discrepancy between two washing procedures with water. For replicate 1 the water-washing procedure might be too aggressive and cause loss of some gliadin proteins because these proteins could be soluble in water by washing with salts (Fu *et al.*, 1996). For replicate 2 the procedure was not effective enough to dissolve all the salt residuals. Thus, difference in the concentrations of crude gliadins in extraction replicates occurred.

4.1.2. Ultrasonic Analyses

4.1.2.1. Ultrasound Velocity of 70% (v/v) Aqueous Alcohol Solutions

In the analyses of serial dilutions of crude gliadins, 70% aqueous alcohol solutions were used as a reference solution. To determine the sensitivity of the ultrasonic resonator to the proteins in the solvents, the same alcohol solvent was used on two separate days. The properties of this solvent were analyzed when the first and the second replicates of each variety were analyzed (on two different days). The values of ultrasound velocity of 70% (v/v) aqueous alcohol used as the reference solvent for 8 serial dilutions were analyzed (Table 4.2).

Table 4.2 Ultrasound velocity of 70% (v/v) aqueous alcohol used as solvents for 8 serial dilutions.

Alcohol Source	Replicate 1 (m·s^{-1})	Replicate 2 (m·s^{-1})
Alcohol 1 (Glenlea)	1404.923 ± 0.001[1]	1405.051 ± 0.001
Alcohol 2 (Katepwa)	1404.915 ± 0.001	1405.105 ± 0.002
Alcohol 3 (AC Reed)	1404.954 ± 0.006	1405.060 ± 0.001
Alcohol 4 (Genesis)	1404.916 ± 0.017	1405.033 ± 0.007

1. mean ± standard deviation (obtained from 60 readings)

The results show that there is very small variation for the 60 readings taken for the solvents. The solvents used for the same replicate (same column) also have repeatable ultrasound velocity values. However, there is poor repeatability between the solvent replicates on the separate days (same row), considering that the difference between them is 0.14 m·s^{-1}, a value that exceeds the accuracy of the instrument (0.05 m·s^{-1}). The reason for this variation might arise from the change in the aqueous alcohol with time. The extractions and measurements for two replicates were performed on different days and the same bottle of 70% (v/v) aqueous alcohol was used. After the experiment for replicate 1 the alcohol was stocked in the same sealed bottle. It is possible that a new equilibrium between evaporation and condensation was achieved in the bottle and small changes occurred in the composition of the liquid. These small changes appear to be enough to cause fluctuations in the ultrasound velocity. It is also noteworthy that the 70% (v/v) aqueous alcohol has additional components (ethanol-63% v/v,

methanol-3.5% v/v, isopropanol-3.5% v/v) which can aggravate the problem in repeatability of ultrasound velocity caused by evaporation. D'Arrigo and Paparelli (1988) reported that the ultrasound velocity of 70% (v/v) aqueous ethanol at 20 °C was 1410.4 m·s^{-1}, which was about 5 m·s^{-1} different from the values obtained in Table 4.2. The impurity of the ethanol used in the preliminary experiments should account for this difference.

4.1.2.2. Ultrasound Velocity of Serial Dilutions of Crude Gliadins in 70% Alcohol

The values of ultrasound velocity of serial dilutions of crude gliadins from different wheat flours are shown in Figure 4.1 to Figure 4.4. Each point in the charts represents the mean value of 60 readings and the error bars are obtained from the standard deviation of corresponding readings.
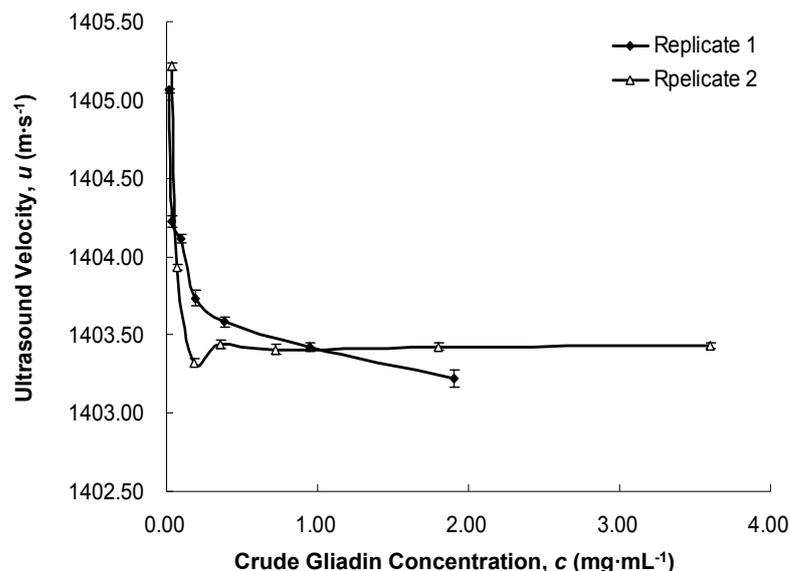
Figure 4.1 Ultrasound velocity values of serial dilutions of gliadins in 70% alcohol from Glenlea.
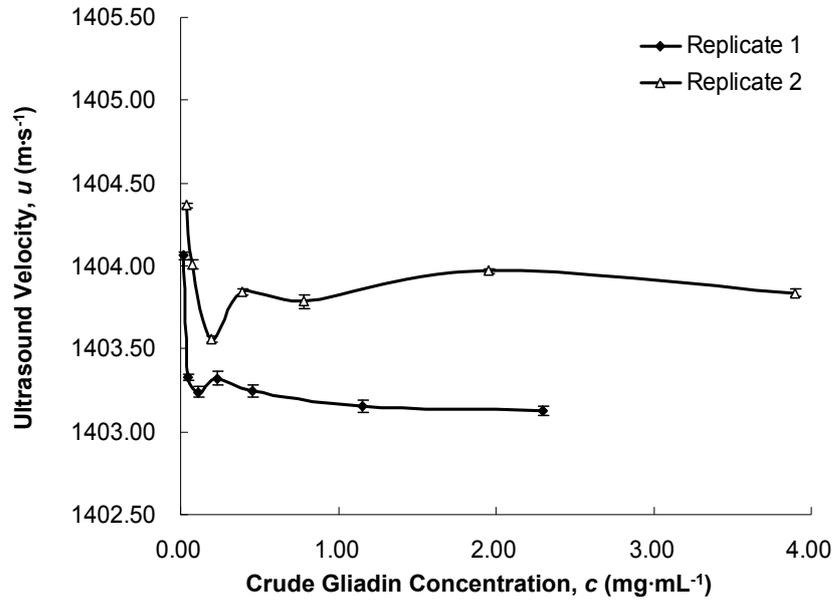
Figure 4.2 Ultrasound velocity values of serial dilutions of gliadins in 70% alcohol from Katepwa.
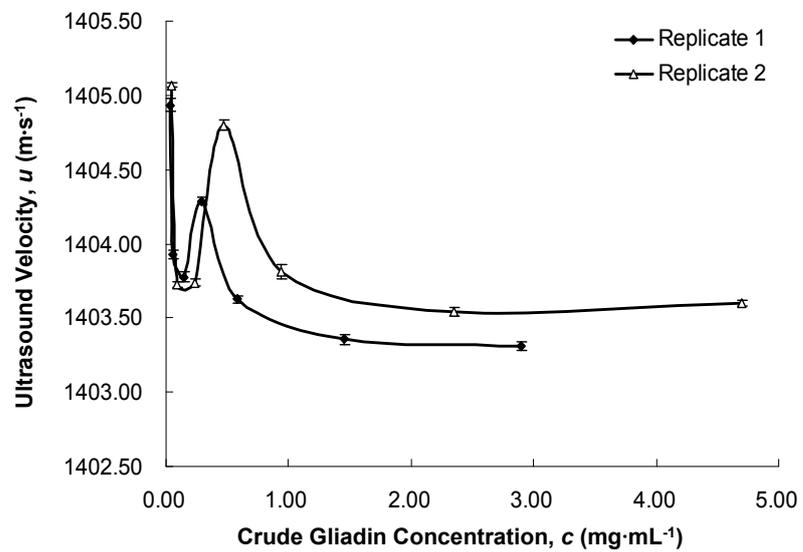
Figure 4.3 Ultrasound velocity values of serial dilutions of gliadins in 70% alcohol from AC Reed.
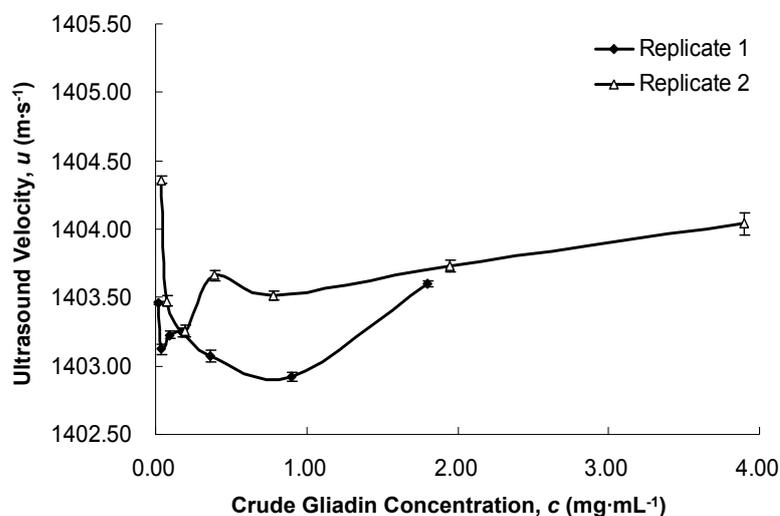
Figure 4.4 Ultrasound velocity values of serial dilutions of gliadins in 70% alcohol from Genesis.

The error bars resulting from 60 readings in the four figures are on the whole concealed by the data points. Except for the samples from Genesis, relatively similar concentration dependences of ultrasound velocities are observed at concentrations larger than 0.5 $mg \cdot mL^{-1}$. Although the two curves in the same figure representing two replicates have similar shapes the repeatability is poor. The poor repeatability in ultrasound velocities could be related to the unknown contamination of the crude gliadins by salt as introduced in *Section 4.1.1*. Besides, the ultrasound velocity shows no linear dependence on protein (crude gliadin) concentration suggesting that the solutions can not be treated as ideal and not suitable for determination of partial specific adiabatic compressibility coefficients (Pavlovskaya *et al*., 1992). This nonideality might result from the contamination of the crude gliadins by salt and the effects of salt on protein structures during the Osborne fractionation (Arrhenius, 1937; Clements, 1973; Fu *et al*., 1996).

4.2. Crude Gliadins Extracted with 70% (v/v) Aqueous Ethanol

Fresh 70% (v/v) alcohol made from pure ethanol (95% v/v) and water based on mass was used for extraction of crude gliadins. This method was selected because salts were not involved and therefore the properties of the solutions of crude gliadins would not be affected by salts. Although the proteins extracted with 70% (v/v) aqueous ethanol directly from flours at room temperature (22 °C) have been reported to be mainly gliadins (Robertson *et al*., 2007), there are some non-protein materials, such as lipids and carbohydrates, that are also extracted as introduced in *Section 2.2.3* (Ponte *et al*., 1967; Hoseney *et al*., 1969b; Békés *et al.*, 1983a; Békés *et al.*, 1983b; Zawistowska *et al.*, 1984). Arrhenius (1937) extracted gliadins from flours with 70% (v/v) EtOH at a temperature of -8 °C to measure the dielectric constant of gliadin solutions. In this part of the preliminary experiment, crude gliadins were extracted with 70% (v/v) EtOH directly from wheat flour at both 23 °C and -8 °C (*Section 3.2.1.2* and *3.2.1.3*) and the protein content of the two extracts were compared to optimize the extraction method of crude gliadins.

The protein contents of crude gliadins extracted at 23 °C and -8 °C were determined (Table 4.3) as introduced in *Section 3.2.5*.

Table 4.3 Protein contents of ethanolic extracted crude gliadins at 23 °C and -8 °C from 1 CWRS flours.

	23 °C	-8 °C
Protein content (%)	74.6 ± 1.3[1]	72.4 ± 1.1

1. mean ± standard deviation (obtained from 3 replicates)

From Table 4.3, the crude gliadins extracted at -8 °C contain no higher amount of proteins than those extracted at 23 °C. Thus, the crude gliadins were extracted with 70% (v/v) EtOH directly from wheat flour and prepared as *Section 3.2.1.2* for further experiments.

4.3. Conclusions of Preliminary Analyses

The 70% (v/v) aqueous alcohols from the modified Osborne fractionation and serial dilutions of gliadin extracts in it have poor repeatability in ultrasound velocity values. The reason might be the complicated composition and the evaporation of this solvent. Fresh 70% (v/v) alcohol directly made from ethanol and water based on mass should be used for further experiments.

Considering the possible contamination of gliadin extracts from salt and the possible effects of salts on the protein structures in Osborne fractionation, 70% (v/v) aqueous ethanol is used to extract crude gliadins. Since the cold extraction method provided no better purity of gliadins, crude gliadins were extracted with 70% (v/v) aqueous ethanol at room temperature (23 °C) for all the following experiments.

5. Solvent Analyses Results and Discussion

The solvent analyses involve the determination of partial specific volumes and partial specific adiabatic compressibility coefficients of crude gliadins in 70% (v/v) EtOH and 4 mM HAc. Aqueous ethanol has been used as a classic solvent for gliadins (Osborne, 1907; Hoseney *et al.*, 1969b; Il *et al.*, 1991) and dilute organic acid is also a good solvent for gliadins (Il *et al.*, 1991). However, the mechanisms of the solubility of gliadins in these two solvents are different (Il *et al.*, 1991; Friedli, 1996). Thus, these two solvents were used to examine effects of molecular structures and their interaction with solvent molecules on the properties of the soluble crude gliadins. Based on these results, the solvent effects on the structures of crude gliadin molecules are discussed.

5.1. Solvent and Treatment Effects on Solubility of Crude Gliadins

The amount of proteins in the most concentrated crude gliadin solutions in different solvents without and with sonication was determined (Table 5.1). The results are obtained from the average values of 3 independent triplicates and there are 3 sub-samples in the mean value of each triplicate.

Table 5.1 Concentration (mg·mL^{-1}) of proteins in different solutions based on micro-Kjeldajl determination.

Solvents	Sonication applied to samples	
	Not applied	60 W × 1.5 min
70% (v/v) Ethanol	9.32 ± 0.25[1]	9.51 ± 0.23
4 mM Acetic Acid	8.68 ± 0.10	8.91 ± 0.14

1. mean ± standard deviation

The variation of the solubility for a given solvent and treatment does not exceed 3% and possibly arises from the discrepancies in the preparation of 3 triplicates as well as the errors in Kjeldahl nitrogen determination (less than 2.5%). The solubility of proteins in 70% (v/v) EtOH is consistently higher than that in 4 mM HAc regardless of the application of sonication treatment. The solubility of crude gliadins in ethanolic solutions (1.05% by weight) is smaller than the value of 4.01% (by weight) in 61.7% (by weight) alcohol reported by Arrhenius (1937). Additionally, Dill and Alsberg (1925) reported that the solubility of gliadins in 70% (v/v) ethanol at room temperature was more than 25% (by weight). Therefore, the precipitates observed in ethanolic and acidic solutions of crude gliadins are not due to saturation of gliadins, but the aggregation of the materials during lyophilisation (as suggested in *Section 3.2.2.3*). The existence of aggregates is evident from the increases in the solubility of proteins in both 70% (v/v) EtOH and 4 mM HAc after sonication treatment (as seen in Table 5.1). The sonication treatment disrupts the aggregates. However, the sonication treatment does not completely destroy the aggregates since there were still non-soluble materials

observed in the solutions after sonication.

It should be also noticed that the materials used are crude gliadins and the word "crude" stresses that there are some non-gliadin proteins (mainly LMW-glutenins) and non-protein materials (such as lipids, carbohydrates, etc.) also dissolved in the solutions. Thus, the amount of soluble materials and the conversion factor for protein to crude gliadins were determined (*Section 3.2.5*). From results of 3 independent triplicates, the protein contents in the soluble materials were determined to be 75% ± 1% for 70% (v/v) EtOH solutions and 79% ± 1% for 4 mM HAc solutions and the sonication treatment had no effect on the protein content of ethanolic and acidic soluble crude gliadins. The components other than proteins in the solutions were not characterized but they were assumed to be mainly lipids according to related references. It has been reported that the co-extracted materials with gliadin proteins from wheat flours are mainly lipids (Cornell *et al.*, 2002). The protein contents of ethanolic soluble protein is close to the results of Ponte *et al*. (1967), who obtained 73.6% proteins and 9.1% lipid from ethanolic extracted crude gliadins. However, in their experiments, the materials for extraction were not flours but glutens. Cornell *et al.* (2002) extracted gliadins directly from wheat flours using 70% (v/v) alcohol and found about 67% protein in the crude gliadin extracts. The higher protein content of ethanolic and acidic soluble crude gliadins compared with the results of Cornell *et al.* (2002) probably results from two reasons: 1) the different protein contents of the wheat flours used in the experiments; and 2) the lyophilization and re-dissolving procedures might improve the protein purity of the crude gliadin solutions.

Unfortunately, Cornell *et al.* (2002) did not report the protein contents of their flours and thus the explanations proposed above can not be confirmed.

5.2. Results of Density and Ultrasound Measurements

The densities of different serial dilutions of crude gliadins were measured using a DMA density meter (*Section 3.2.4*). Values of ultrasound velocity, as well as attenuation, of the serial dilutions were determined with a ResoScan System (*Section 3.2.3*). The ultrasound attenuation of samples is expressed as a/f^2, where a is the ultrasound attenuation coefficient (in m^{-1}) and f (in Hz) is the ultrasound frequency used by the instrument to determine the ultrasound velocity. The frequencies for the measurements of the serial dilutions in 70% (v/v) EtOH and 4 mM HAc were 7.37 MHz and 7.75 MHz, respectively.

The values of density and the ultrasound velocity for 70% (v/v) ethanol at 20 °C were 885.2 ± 0.1 mg·mL^{-1} and 1410.2 ± 0.4 m·s^{-1}, respectively, and they were near to the results of D'Arrigo and Paparelli (1988), who obtained these two parameters as 884.1 mg·mL^{-1} and 1410.4 m·s^{-1}, respectively. The density and the ultrasound velocity for 4 mM HAc at 20 °C were 998.37 ± 0.01 mg·mL^{-1} and 1482.50 ± 0.09 m·s^{-1}, respectively. Unfortunately, no reference data of the density and the ultrasound velocity for 4 mM HAc are available. However, the ultrasound velocity and density values of 4 mM HAc solutions are very similar to water and have very good repeatability.

Like the definition of specific viscosity (Moore, 1976), specific values are used as measures of the relative fractional change in the density and ultrasound

properties of solutions by adding the solutes. The specific density, ρ_{sp}, specific

ultrasonic velocity, u_{sp}, and specific attenuation a_{sp} are calculated as

$$\rho_{sp} = \rho/\rho_o - 1, \; u_{sp} = u/u_o - 1, \text{ and } \; a_{sp} = \frac{a}{a_o} - 1 = \frac{\left(a/f^2\right)}{\left(a/f^2\right)_o} - 1,$$ where the subscript $_o$

stands for the properties of the bulk solvents and all the values of ρ, u and a

were directly obtained from experimental data. The values of specific density,

specific ultrasound velocity and specific ultrasound attenuation are plotted against

crude gliadin concentration in Figures 5.1 to 5.3.
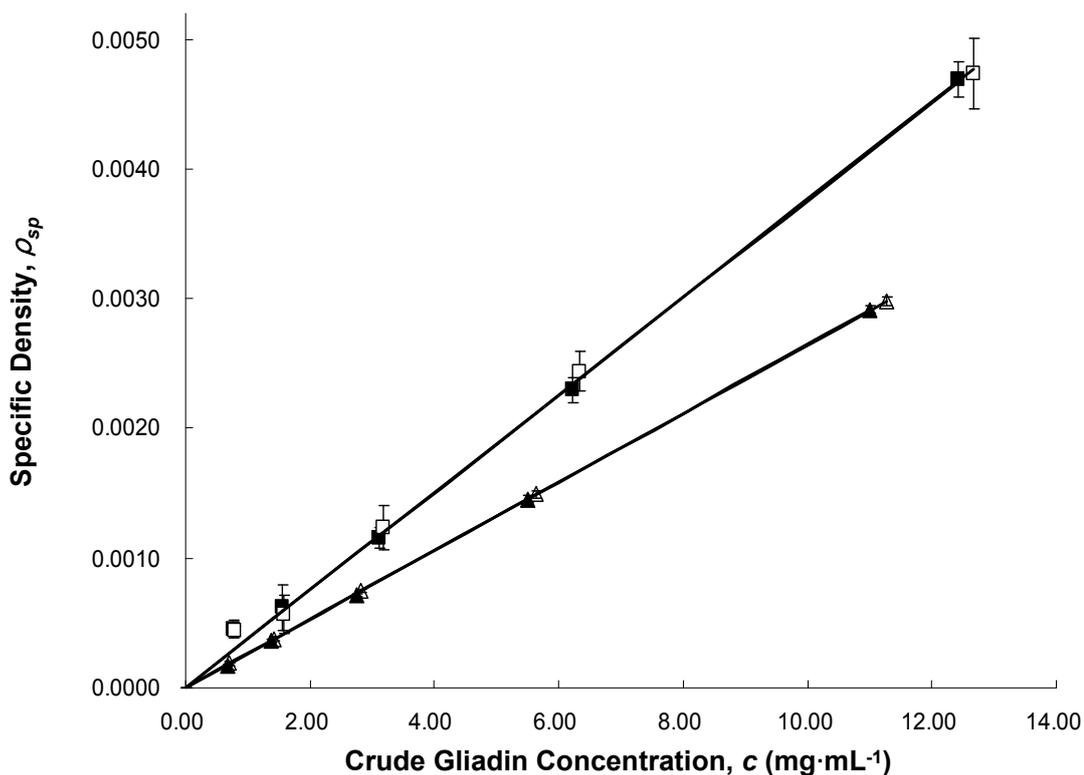
Figure 5.1 Specific density, ρ_{sp}, of serial dilutions of crude gliadins in 70% (v/v) EtOH (■), sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).
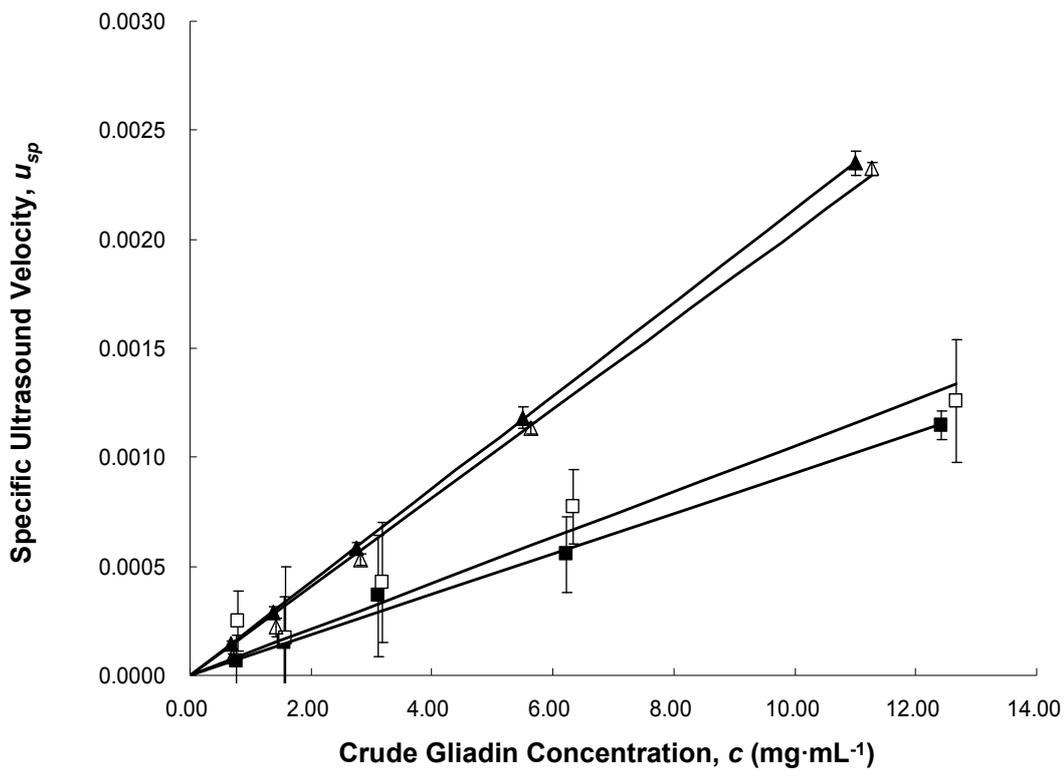
Figure 5.2 Specific ultrasonic velocity, u_{sp}, of serial dilutions of crude gliadins in 70% (v/v) EtOH (■), sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).
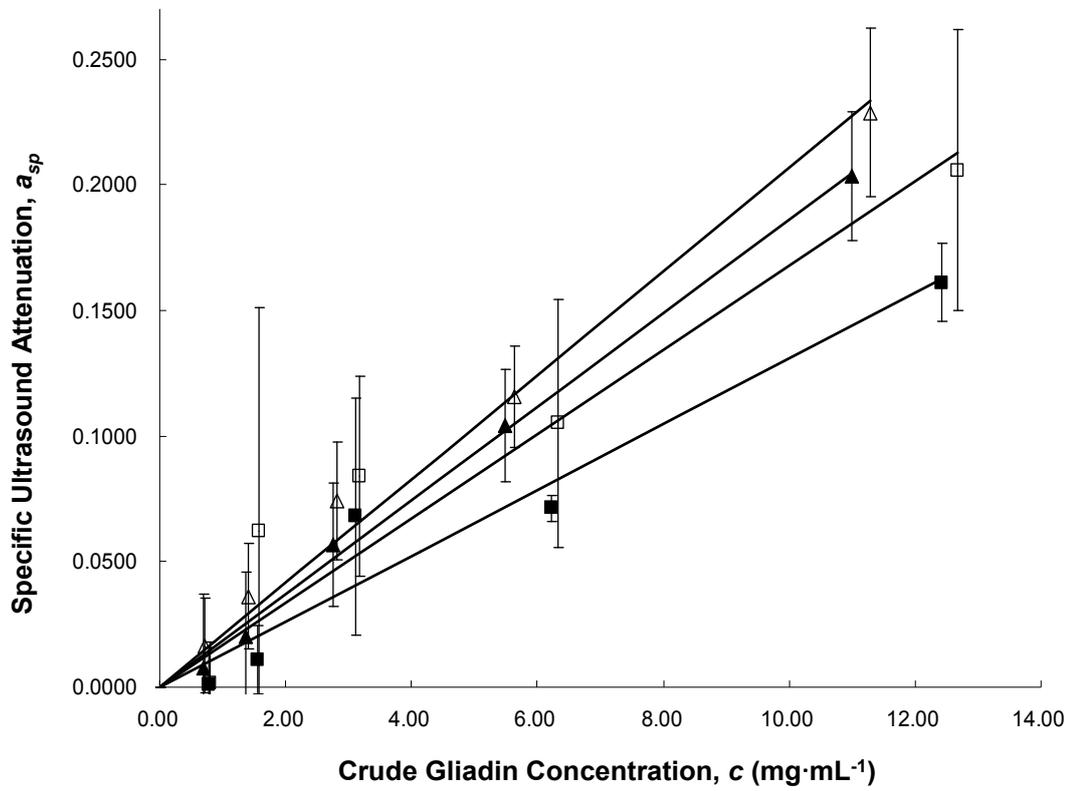
Figure 5.3 Specific ultrasonic attenuation, a_{sp}, of serial dilutions of crude gliadins in 70% (v/v) EtOH (■), sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).

Generally, the specific density, ρ_{sp}, specific ultrasound velocity, u_{sp}, and specific attenuation, a_{sp}, have good linear relationships ($R^2 > 0.92$) with crude gliadin concentrations in all the solutions. Positive effects of crude gliadins on the density, ultrasound velocity and ultrasound attenuation of the solutions are observed. Larger error bars, especially for the ethanolic extracts, are observed in the determinations of ultrasound properties, u_{sp}, and a_{sp} compared to ρ_{sp}, indicating that ultrasound measurements are more sensitive to the properties of protein solutions than density measurements (Chalikian *et al*. 1996). Besides, the two curves representing the same solvent but different treatments (without or with sonication) almost overlap in Figure 5.1 but obviously separate in Figure 5.2 and Figure 5.3 indicating that effects of sonication treatment are less on the density than on the ultrasound properties of the solutions.

According to the definition of specific quantities as introduced above, the intercepts of the regression lines of ρ_{sp}, u_{sp}, and a_{sp}, in Figure 5.1, Figure 5.2 and Figure 5.3 are set to be 0. The modified regression lines (with intercept at 0) still have good R^2 values (> 0.89) but somewhat smaller than unmodified ones ($R^2 > 0.92$). Some points are observed to deviate from the regression lines in the charts, especially for the differently treated ethanolic extracts at low concentrations. One reason might be the relatively strong evaporation of the solvent. The most dilute solutions were made at the end of the preparation of serial dilutions and more ethanol might evaporate from the solvent, so the specific densities of the most dilute solutions tend to be relatively large. Since the linearity of ρ_{sp}, u_{sp}, and a_{sp} with concentration suggests that there is no aggregation or

variation of the solutes and the solutions can be treated as ideal (Pavlovskaya *et al.*, 1992), it is reasonable to assume that besides the evaporation of the ethanol, changes in the solute properties resulting from dilution might occur in the crude gliadin solutions and cause the deviations of these points, a finding that will be discussed later. The concentration dependences of specific density, specific ultrasound velocity and specific ultrasound attenuation of crude gliadins in different solutions ($d\rho_{sp}/dc$, du_{sp}/dc, and da_{sp}/dc), which numerically equal the slopes ($[\rho]_{sp}$, $[u]_{sp}$, and $[a]_{sp}$) of the plots of ρ_{sp}, u_{sp}, and a_{sp} against concentration *c*, and the R^2 value of each regression line, are shown in Tables 5.2 to 5.4.

Table 5.2 Concentration dependence of specific density, $[\rho]_{sp}$, of different serial dilutions of crude gliadins.

Solvent	Sonication	$[\rho]_{sp}$ $\times 10^{-4}$ (mL·mg^{-1})	R^2
70% (v/v) EtOH	Not applied	3.764	0.998
	60 W × 1.5 min	3.769	0.998
4 mM HAc	Not applied	2.649	1.000
	60 W × 1.5 min	2.640	1.000

Table 5.3 Concentration dependence of specific ultrasound velocity, $[u]_{sp}$, of different serial dilutions of crude gliadins.

Solvent	Sonication	$[u]_{sp}$ ×10^{-4} (mL·mg^{-1})	R^2
70% (v/v) EtOH	Not applied	0.929	0.991
	60 W × 1.5 min	1.059	0.933
4 mM HAc	Not applied	2.138	1.000
	60 W × 1.5 min	2.033	0.997

Table 5.4 Concentration dependence of specific ultrasound attenuation, $[a]_{sp}$, of different serial dilutions of crude gliadins.

Solvent	Sonication	$[a]_{sp}$ (mL·mg^{-1})	R^2
70% (v/v) EtOH	Not applied	0.0130	0.937
	60 W × 1.5 min	0.0170	0.892
4 mM HAc	Not applied	0.0186	0.997
	60 W × 1.5 min	0.0207	0.989

From Tables 5.2 to 5.4, dilute acidic solutions without sonication are supposed to be the most ideal solutions because of the largest R^2 values for regression lines of ρ_{sp}, u_{sp}, and a_{sp} against solute concentration (Pavlovskaya *et al.*, 1992). Sonication is observed to have no effects on densities of different solutions. It is untrustworthy to conclude that the sonication treatment has effects on ultrasound velocities of ethanolic solutions because of the large error bars for ethanolic dilutions in Figure 5.2. However, it is safe to state that sonication treatment has small but decreasing effects on ultrasound velocities of crude gliadins in dilute acidic solutions.

5.3. Specific Volume of Crude Gliadins

The apparent specific volume of crude gliadins in solutions, $\bar{v}$, is calculated from the density and the solute concentration of the solutions as $\bar{v} = \dfrac{1-(\rho-c)/\rho_o}{c}$ (Equation 2 in *Section 2.1.1.1*) and the results are shown in Figure 5.4.
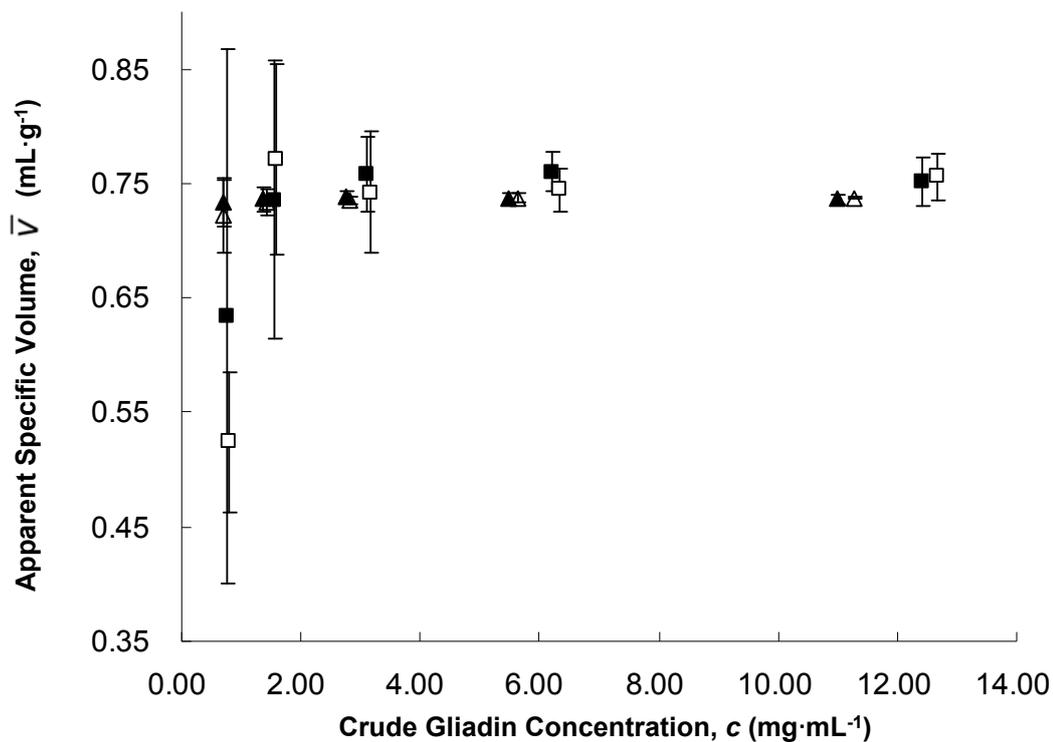
Figure 5.4 Apparent specific volume, $\overline{V}$, of crude gliadins in 70% (v/v) EtOH (■),
sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲)
and sonicated ones in 4 mM HAc (△).

It can be seen that different values are observed for the most dilute ethanolic

solutions without or with sonication treatment and the corresponding error bars

increase dramatically, outcomes which are consistent with the observations in

ρ_{sp} (Figure 5.1). Without the results of the most dilute ethanolic solutions, Figure

5.4 shows that the apparent specific volume is independent of concentration and

the values are alike for crude gliadins in the different solutions.

The specific density values, ρ_{sp}, of the crude gliadins in the most dilute

ethanolic solutions, both without and with sonication treatment, are observed to deviate from their regression lines as shown in Figure 5.1, indicating that the solutes in these very dilute solutions have different molecular states from those in concentrated ones (Pavlovskaya *et al.*, 1992). Thus, the extrapolations for partial specific volumes, $\bar{v}^o$, of crude gliadins in ethanolic solutions are performed using the values of the apparent specific volume obtained from the four most concentrated dilutions and the results are shown in Table 5.5.

Table 5.5 Partial specific volume, $\bar{v}^o$, of crude gliadins in different solutions.

Solvent	Sonication	$\bar{v}^o$ (mL·g^{-1})
70% (v/v) Ethanol[1]	Not applied	0.747
	60 W × 1.5 min	0.757
4 mM Acetic Acid	Not applied	0.736
	60 W × 1.5 min	0.729

1. Partial specific volume, $\bar{v}^o$, of crude gliadins in ethanolic solutions are obtained by extrapolating the first four concentrated points in Figure 5.4.

Considering the size of the error bars in Figure 5.4, the partial specific volumes, $\bar{v}^o$, crude gliadins are not affected by sonication treatment either in aqueous ethanol or in dilute acetic acid, but it is correct to conclude that crude gliadins have larger partial specific volume in ethanolic solutions than in acetic acid. The values of $\bar{v}^o$ of the crude gliadins in 4 mM HAc, with and without sonication

treatment, are similar to those obtained by Krejci and Svedberg (1935) who reported the partial specific volume of gliadins ranged from 0.712 to 0.736 mL·g^{-1} in 8 mM HCl – 8 mM KCl aqueous solutions.

The results of $\bar{v}$ and $\bar{v}^o$ give some information on the molecular states of crude gliadins in the solutions, such as the intrinsic and hydration properties of the solutes, and this will be discussed later accompanied with the results of compressibility, as the latter provide more details on the hydration states of the dissolved molecules (Chalikian *et al*., 1996; Chalikian, 2001).

5.4. Compressibility of Crude Gliadins

The adiabatic compressibility coefficient, β_S, is calculated from the values of density and ultrasound velocity of the solutions as $\beta_S = \dfrac{1}{\rho u^2}$ (Equation 11 in *Section 2.1.2.3*). The values of relative adiabatic compressibility coefficient, the ratio of the values of solution to that of solvent, β_S / β_{So}, are shown in Figure 5.5.
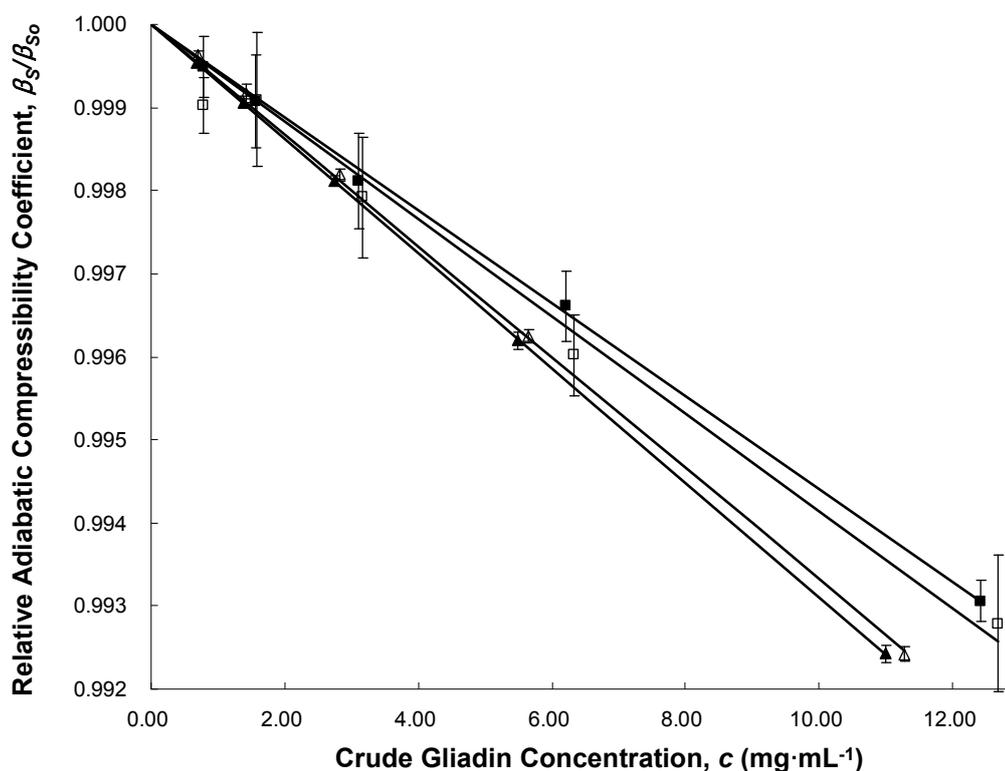
Figure 5.5 Relative adiabatic compressibility coefficient, β_S/β_{So}, of crude gliadins in 70% (v/v) EtOH (■), sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).

Results show that the adabatic compressibility coefficients of the solutions decrease with increasing concentration of crude gliadins in the solutions. The intercepts of the regression lines in Figure 5.5 are set at 1 according to the meaning of the expression of β_S/β_{So}. Good linearity ($R^2 > 0.98$) of β_S/β_{So} with the crude gliadin concentration in different solutions is observed. Similar to the results of ρ_{sp} and u_{sp}, relative adiabatic compressibility coefficients, β_S/β_{So} of crude gliadins in dilute ethanolic solutions, especially in the sonicated ethanolic

solutions, are observed to deviate from the regression lines. These deviations might suggest that dilution affects the crude gliadins and the molecular states of solutes in these dilute solutions are different from those in concentrated ones (Pavlovskaya *et al.*, 1992).

The partial specific adiabatic compressibility coefficient, $\overline{\beta}_S^o$, is calculated as

$$\overline{\beta}_S^o = \left(\frac{\beta_{So}}{\overline{v}^o}\right) \times \lim_{c \to 0} \frac{\beta_S/\beta_{So} - \Phi_o}{c}$$ (Equation 16 in *Section 2.1.2.3*) where the

volumetric fraction of the solvent, $\Phi_o = \dfrac{\rho - c}{\rho_o}$ (*Section 2.1.1.1*). The adiabatic

compressibility coefficient of the solvent, β_{So} (5.68 $\times 10^{-5}$ bar^{-1} for 70% aqueous ethanol and 4.56 $\times 10^{-5}$ bar^{-1} for 4 mM acetic acid), as well as the partial specific volume of crude gliadins, $\overline{v}^o$ (Table 5.5), is constant. Thus, the values of $\overline{\beta}_S^o$ are

determined by extrapolating the expression $\dfrac{\beta_S/\beta_{So} - \Phi_o}{c}$ to zero concentration.

The values of $\dfrac{\beta_S/\beta_{So} - \Phi_o}{c}$ are plotted against concentration of crude gliadins in

Figure 5.6.
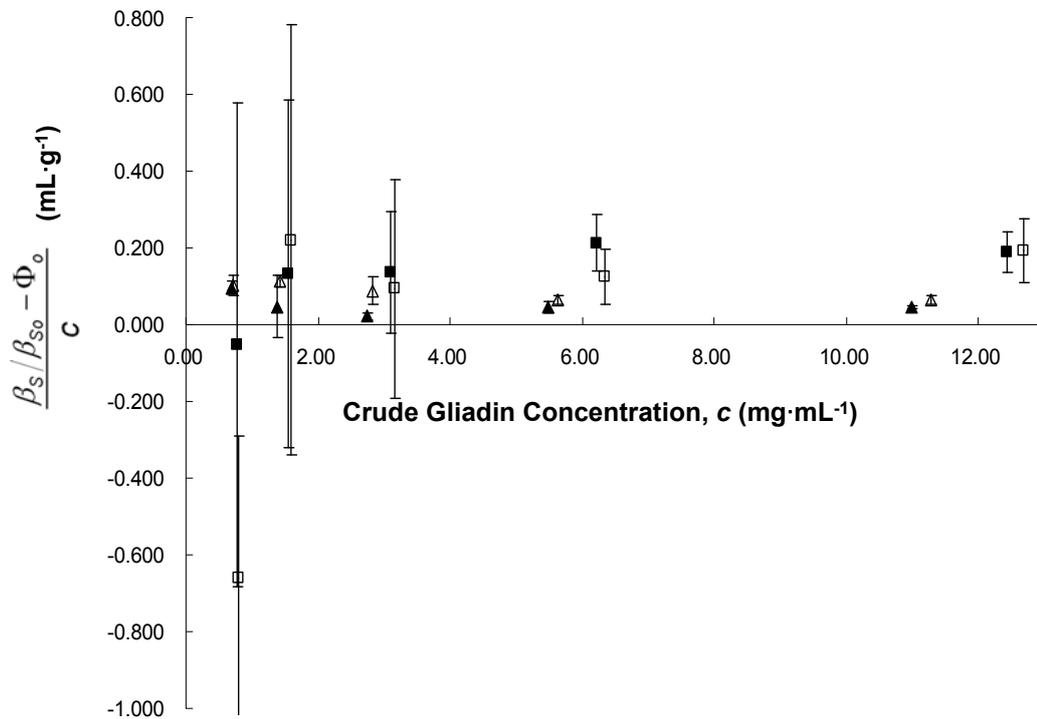
Figure 5.6 Values of the expression, $\dfrac{\beta_S / \beta_{So} - \Phi_o}{c}$, of crude gliadins in 70% (v/v) EtOH (■), sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).

Like the results of apparent specific volumes, the values of $\dfrac{\beta_S / \beta_{So} - \Phi_o}{c}$ have large error bars for dilute ethanolic solutions and the values are unexpectedly small at the lowest concentrations in ethanolic solutions either without or with sonication treatment. It has been assumed that the states of the crude gliadin molecules in these dilute ethanolic solutions are different from those in concentrated ones, so the zero extrapolations for $\displaystyle\lim_{c \to 0} \dfrac{\beta_S / \beta_{So} - \Phi_o}{c}$ are performed

using the values of the four most concentrated ethanolic solutions. The partial

specific adiabatic compressibility coefficients, $\bar{\beta}_S^o$, of crude gliadins are

calculated based on the results of $\lim\limits_{c \to 0} \dfrac{\beta_S/\beta_{So} - \Phi_o}{c}$, β_{So}, and $\bar{v}^o$. The results are

shown in Table 5.6.

Table 5.6 Partial specific volume, $\bar{v}^o$, values of $\lim\limits_{c \to 0} \dfrac{\beta_S/\beta_{So} - \Phi_o}{c}$, and partial specific adiabatic compressibility coefficient,

$\bar{\beta}_S^o$, of crude gliadins in different solutions.

Solvents	Sonication	$\bar{v}^o$ (mL·g^{-1})	$\lim\limits_{c \to 0} \dfrac{\beta_S/\beta_{So} - \Phi_o}{c}$ (mL·g^{-1})	$\bar{\beta}_S^o$ (×10^{-6} bar^{-1})
70% (v/v) Ethanol[1]	Not applied	0.747	0.135	10.27
	60 W × 1.5 min	0.757	0.148	11.10
4 mM Acetic Acid	Not applied	0.736	0.060	3.72
	60 W × 1.5 min	0.729	0.105	6.57

1. Values of $\bar{v}^o$ and $\lim\limits_{c \to 0} \dfrac{\beta_S/\beta_{So} - \Phi_o}{c}$ of crude gliadins in ethanolic solutions are obtained by extrapolating the first four concentrated points in Figure 5.4 and Figure 5.6.

Considering the size of the error bars in Figure 5.6, the partial specific adiabatic compressibility coefficient, $\overline{\beta}_S^o$, of crude gliadins in ethanolic solutions is not affected by sonication treatment. The effect of sonication on $\overline{\beta}_S^o$ of crude gliadins in 4 mM HAc is strong, with compressibility increasing with sonication.

From the definition of partial specific adiabatic compressibility coefficient,

$$\overline{\beta}_S^o = \frac{\overline{K}_S^o}{\overline{V}^o}$$ (Equation 13 in *Section 2.1.2.3*) and the equation

$$\overline{\beta}_S^o = \left(\frac{\beta_{So}}{\overline{V}^o}\right) \times \lim_{c \to 0} \frac{\beta_S / \beta_{So} - \Phi_o}{c}$$ (Equation 16 in *Section 2.1.2.3*), the partial specific

adiabatic compressibility of the protein, $\overline{K}_S^o = \beta_{So} \times \lim_{c \to 0} \dfrac{\beta_S / \beta_{So} - \Phi_o}{c}$, can be

obtained. Based on the relationship between the apparent specific adiabatic compressibility and the partial specific adiabatic compressibility, $\overline{K}_S^o = \lim_{c \to 0} \overline{K}_S$

(Equation 14 in *Section 2.1.2.3*), the apparent specific adiabatic compressibility is

obtained as $\overline{K}_S = \beta_{So} \times \dfrac{\beta_S / \beta_{So} - \Phi_o}{c}$. The results of apparent specific adiabatic

compressibility, $\overline{K}_S$, of crude gliadins in different serial dilutions are plotted against crude gliadin concentration in Figure 5.7.
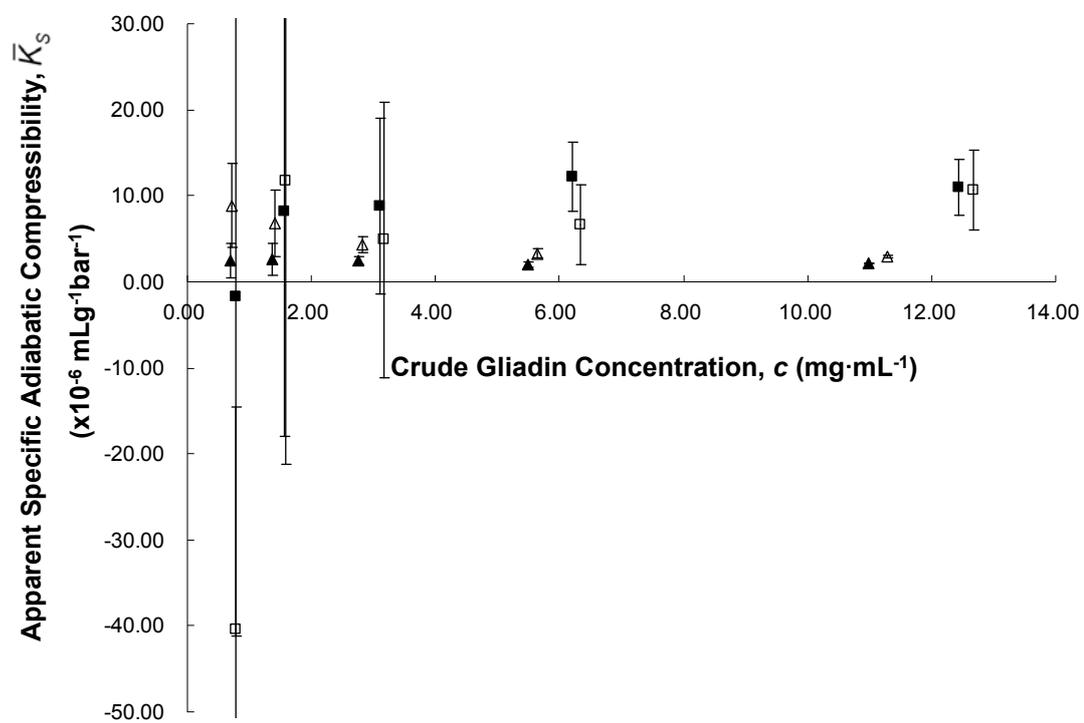
Figure 5.7 Apparent specific adiabatic compressibility, $\bar{K}_S$, of crude gliadins in 70% (v/v) EtOH (■), sonicated ones in 70% (v/v) EtOH (□); crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).

5.5. Discussions on Solvent Analyses

The molecular states including interior structures and hydration properties of dissolved crude gliadins are discussed in this section based on the values of apparent specific volume, $\bar{v}$, partial specific volume, $\bar{v}^o$, apparent specific adiabatic compressibility, $\bar{K}_S$, and partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, as these parameters provide enough information to estimate the intrinsic and hydration properties of protein molecules. It has been reviewed in *Chapter 2* that $\bar{v}^o$, as well as $\bar{v}$, provides straightforward information on the intrinsic structures of proteins, as the partial specific volume, $\bar{v}^o$, mainly depends on the intrinsic volume, the sum of the Van der Waals volume of all constituent atoms, v_W, and the total volume of the voids inside the protein molecule, v_v (Chalikian *et al.*, 1996; Taulier and Chalikian, 2002; Mori *et al.*, 2006). On the other hand, the apparent specific adiabatic compressibility, $\bar{K}_S$, of the proteins in one given solution is sensitive to both intrinsic packing and molecular surface hydration properties of the proteins at the corresponding concentration (Gekko and Noguchi, 1979; Gekko and Hasegawa, 1986; Chalikian *et al.*, 1996). In this section, the effects of different solvents and sonication treatment on the molecular states of the dissolved crude gliadins, such as the imperfect packing of the atoms inside the globular proteins and the types of hydrations (charged, polar and non-polar) on the molecular surface, are estimated based on the values of the partial specific volume, $\bar{v}^o$, and the partial specific adiabatic compressibility

coefficient, $\bar{\beta}_S^o$, and the effects of dilution on the crude gliadins in different solutions are discussed based on the apparent specific volume, $\bar{v}$, and the apparent specific adiabatic compressibility, $\bar{K}_S$.

5.5.1. Crude Gliadins in Different Solvents

From Table 5.6, the partial specific volume, $\bar{v}^o$, of crude gliadins obtained from high concentrated ethanolic solution (the first 4 concentrated points) is larger than that in dilute acidic solutions regardless of the sonication treatment. Békés *et al.* (1983a, 1983b, 1992) have reported that proteins (LMW-glutenins and gliadins) aggregate with lipids in ethanol soluble gliadin-rich fractions, and Pomeranz and Chung (1978) and McCann *et al.* (2009) have reported that polar lipids, especially phospholipids, associate with gliadins through hydrogen bonds and hydrophobic interactions and these lipids dissociate from gliadins in the presence of dilute acid. The large values of $\bar{v}^o$ of crude gliadins in 70% (v/v) EtOH might be due to increased solvent inaccessible void space between gliadins and lipids acting in a complex, exerting an effect of increasing intrinsic volume of the complexes. Conversely, the protein-lipid complexes appear to be absent in 4 mM HAc, as suggested by the small value of $\bar{v}^o$ of crude gliadins in 4 mM HAc.

Crude gliadins in 4 mM HAc have smaller values of partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ (Table 5.6). One reason might be that crude gliadins in 4 mM HAc have more charged hydrations because the net charges of the protein molecules are increased in dilute acid (Damodaran, 1996). Crude

gliadins in 70% (v/v) EtOH might interact mainly with ethanol molecules through hydrophobic interactions (Damodaran, 1996; McCann *et al*., 2009). Thus, the crude gliadins in 4mM HAc have small value of $\bar{\beta}_S^o$ because of more negative contribution from charged hydrations (Kharakoz, 1991; Chalikian *et al.*, 1996; Taulier and Chalikian, 2002). However, this reason is not strong enough to explain the remarkable difference between the compressible properties of crude gliadins in 4 mM HAc and 70% (v/v) EtOH. According to $\bar{K}_S^o = \left(\bar{K}_M^o + \Delta\bar{K}_I^o \right)_S$ (Equation 22 in *Section 2.1.3.1*), there are two properties of proteins causing changes in molecular compressible properties. Beside the interactions of crude gliadins with solvent molecules in the two solutions [mainly charged and polar hydrations with water in 4 mM HAc, and mainly hydrophobic interactions with ethanol in 70% (v/v) EtOH], different intrinsic structure of the solutes should be considered. As suggested before, protein-lipid complexes in 70% (v/v) EtOH have more solvent inaccessible void space than gliadins and this increased void space increases the intrinsic compressibility of the crude gliadins in aqueous ethanolic solutions (Gekko and Noguchi, 1979; Gekko and Hasegawa, 1986; Gekko and Yamagami, 1991; Chalikian *et al*., 1994a; Andrews *et al*., 2002).

Values of $\bar{v}$ and $\bar{K}_S$ of crude gliadins in unsonicated ethanolic and dilute acidic solutions are shown in Figure 5.8 and Figure 5.9 for higher resolution. The dilution effects on the proteins are discussed based on $\bar{v}$ and $\bar{K}_S$.
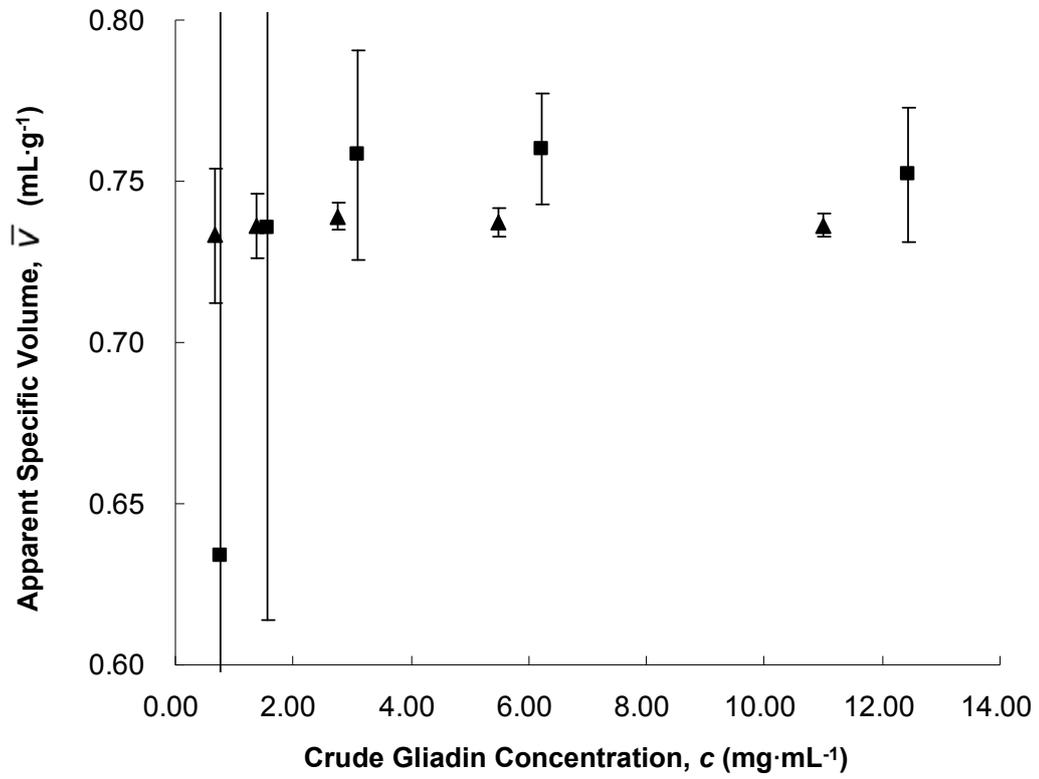
Figure 5.8 Apparent specific volume, $\bar{v}$, of crude gliadins in unsonicated 70% (v/v) EtOH (■) and in unsonicated 4 mM HAc (▲).
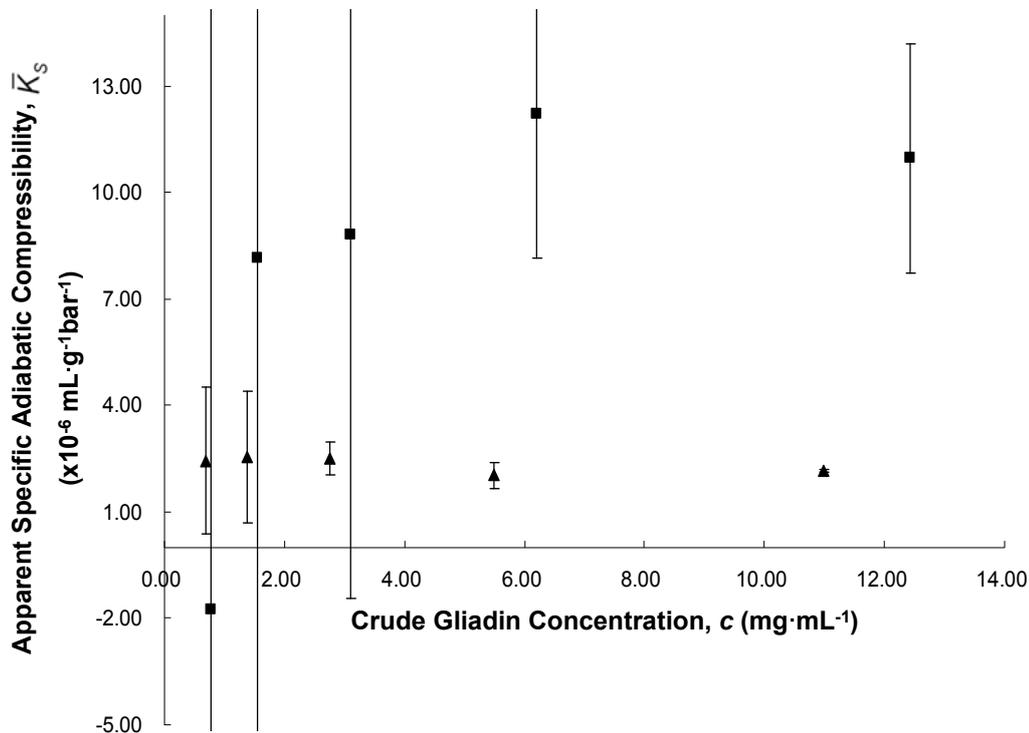
Figure 5.9 Apparent specific adiabatic compressibility, $\bar{K}_S$, of crude gliadins in unsonicated 70% (v/v) EtOH (■) and in unsonicated 4 mM HAc (▲).

From Figure 5.8 and Figure 5.9, the apparent specific volume, $\bar{v}$ and the apparent specific adiabatic compressibility, $\bar{K}_S$, of crude gliadins in unsonicated dilute acidic (4 mM HAc) solutions are independent of concentrations of the solutions. The values of $\bar{v}$ and $\bar{K}_S$ of crude gliadins in unsonicated alcoholic (70% EtOH) solutions, except at the lowest concentration, are considered as independent of solution concentrations. However, both $\bar{v}$ and $\bar{K}_S$ of crude gliadins in the most dilute unsonicated alcoholic solution tend to have smaller values than at the other concentrations.

At the lowest concentration, crude gliadins in the ethanolic solution tend to have smaller apparent specific volume than those at higher concentrations as well as those in acetic acid solutions. One possible reason is the degradation of protein-lipid complexes caused by dilution. Ethanolic soluble crude gliadins at the lowest concentration are observed to have a small value of apparent specific adiabatic compressibility, which might be evidence of the disruption of complexes in the most dilute ethanolic solution. As the lipids dissociate from gliadins, inter-molecular solvent inaccessible void space inside the protein-lipid complexes decreases or disappears and the value of $\bar{v}$ of crude gliadins decreases. Similarly, the intrinsic compressibility decreases because of the loss of the solvent inaccessible void space and moreover, the solvent accessible surface of solute molecules increases which together results in a decreased apparent specific adiabatic compressibility. However, the degradation of protein-lipid complexes can not explain the decrease in $\bar{v}$ and $\bar{K}_S$ of crude gliadins in the most dilute ethanolic solution, which are both smaller than those of crude gliadins in 4 mM HAc. Another reason causing the small values of $\bar{v}$ and $\bar{K}_S$ of crude gliadins in the ethanol solution at the lowest concentration might be the denaturation of the proteins. As introduced in *Section 2.1.3.3*, partial or full unfolding of globular protein is accompanied by decreases in apparent specific adiabatic compressibility values (Chalikian *et al.*, 1995). Unfortunately, there is no research on dilution effect of aqueous ethanol on protein structures and conformations. Discussion on the degradation of protein-lipid complexes and denaturation of the proteins in the ethanol solution at low concentrations is thus speculative.

5.5.2. Sonicated Crude Gliadins

The sonication treatment is observed to have a positive effect on the solubility of crude gliadins in both 70% (v/v) EtOH and 4 mM HAc (Table 5.1). Sonication might disturb the structures of the crude gliadins in different solutions. The possible effects of sonication are discussed in this section based on the volumetric ($\bar{v}^o$ and $\bar{v}$) and compressible ($\bar{\beta}_S^o$ and $\bar{K}_S$) properties of crude gliadins.

5.5.2.1. Sonication Effects on Crude Gliadins in 70% (v/v) EtOH

From Table 5.6, the partial specific volumes, $\bar{v}^o$, of crude gliadins obtained from high concentrated (the first 4 concentrated points) unsonicated and sonicated ethanolic solutions are not different from each other but the value of $\bar{v}^o$ of sonicated crude gliadins tends to be higher. Similar results are found for the partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ of ethanolic soluble crude gliadins, i.e., the values of $\bar{\beta}_S^o$ of crude gliadins in unsonicated and sonicated ethanolic solutions are near to each other but the latter tend to be larger.

It is considered that sonication treatment disrupts the aggregates of crude gliadins formed during lyophilization and increases the solubility of crude gliadins in ethanolic solutions (*Section 5.1*). It is possible that the sonication also disrupts the protein-lipid complexes dissolved in 70% (v/v) EtOH. Based on the results of $\bar{v}^o$ and $\bar{\beta}_S^o$, the formation of the protein-lipid complexes in ethanolic solutions is

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observed to be reversible as reported by Békés *et al*. (1983b). Thus, sonication treatment is considered to have no effects on protein-lipid complexes in ethanolic solutions. The possible reason for the increasing tendency of $\bar{v}^o$ and $\bar{\beta}_S^o$ for sonicated crude gliadins is the disturbing effect of sonication on the intrinsic structures of globular proteins. Güzey *et al*. (2006) found that sonication treatment increased the surface activity of bovine serum albumin (BSA) but it did not denature the proteins. According to the relationship of the partial specific adiabatic compressibility coefficients and the functionalities of proteins (*Section 2.1.3.4*), the conclusion of Güzey *et al*. (2006) is consistent with the present experimental results that sonication treatment increased the partial specific adiabatic compressibility coefficient of crude gliadins. As introduced by Chalikian *et al*. (1995), the partial specific adiabatic compressibility coefficient of globular proteins increased during the transition from the native (N) state to the compact intermediate (CI) state. The solvent inaccessible core inside a protein molecule, stabilized by hydrophobic interactions, might be disturbed by sonication and a CI state with a looser intrinsic structure occurs. However, these undenatured gliadins might be further affected by dilution in ethanolic solutions.

Values of $\bar{v}$ and $\bar{K}_S$ of differently treated (without and with sonication) crude gliadins in 70% (v/v) EtOH are shown in Figure 5.10 and Figure 5.11 and the effects of dilution are discussed.
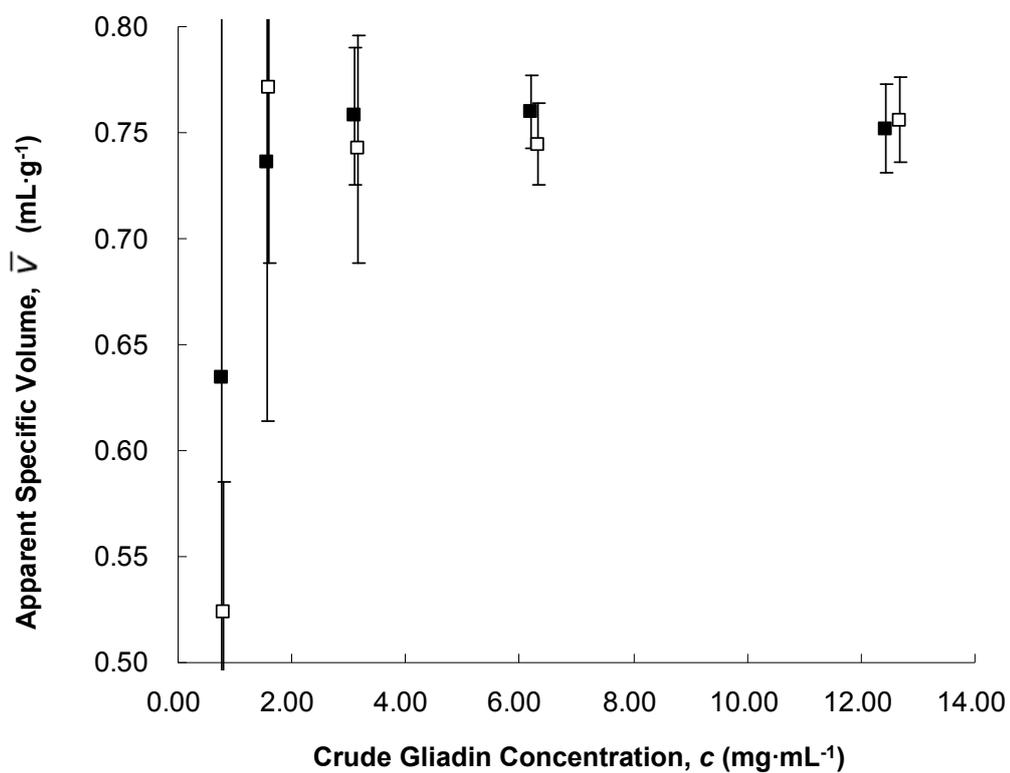
Figure 5.10 Apparent specific volume, $\bar{v}$, of crude gliadins in 70% (v/v) EtOH (■), and sonicated ones in 70% (v/v) EtOH (□).
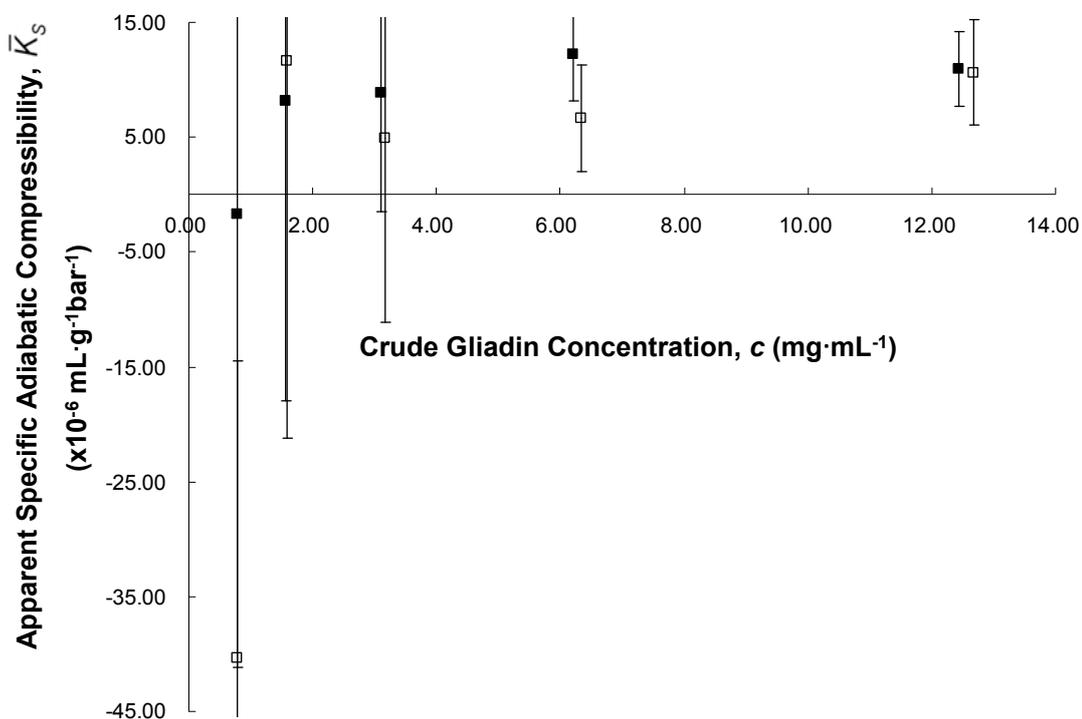
Figure 5.11 Apparent specific adiabatic compressibility, $\bar{K}_S$, of crude gliadins in 70% (v/v) EtOH (■) and sonicated ones in 70% (v/v) EtOH (□).

From Figure 5.10, the apparent specific volumes $\bar{v}$ of unsonicated crude gliadins at high concentrations (the first 4 concentrated points) in ethanolic solutions are not different from sonicated ones and the values for unsonicated and sonicated samples are independent of solution concentrations. However, smaller values of $\bar{v}$ of crude gliadins at the lowest concentration in both unsonicated and sonicated alcoholic solutions are observed. Similar results of the apparent specific adiabatic compressibility, $\bar{K}_S$, are obtained as seen in Figure 5.11. Both $\bar{v}$ and $\bar{K}_S$ of sonicated ethanolic soluble crude gliadins tend to be smaller than those of

unsonicated ones in the most dilute ethanolic solutions.

As discussed in *Section 5.5.1*, degradation of protein-lipid complexes and denaturation of gliadins might account for the dramatic decrease in $\bar{v}$ and $\bar{K}_S$ of crude gliadins in the most dilute unsonicated ethanolic solutions. This speculation can be used to explain the similar decrease in $\bar{v}$ and $\bar{K}_S$ of crude gliadins in the most dilute sonicated ethanolic solutions. The smaller values of $\bar{v}$ and $\bar{K}_S$ of crude gliadins in the most dilute sonicated ethanolic solution in Figure 5.10 and Figure 5.11 might be caused by a greater degree of unfolding of the globular proteins. According to Chalikian *et al.* (1995), the partial specific adiabatic compressibility of a protein decreases dramatically from its partial folding state to its fully folding state. The crude gliadins solubilized with sonication might be easier to unfold than the ones without sonication (Güzey *et al.*, 2006) and therefore, the former have a greater degree of unfolding at low concentrations in ethanolic solutions. The reason this occurs might be the disruption of the intrinsic structures of globular proteins by sonication as speculated in terms of the $\bar{v}^o$ and $\bar{\beta}_S^o$ results.

5.5.2.2. Sonicated Crude Gliadins in 4 mM HAc

From Table 5.6, the partial specific volumes, $\bar{v}^o$, of crude gliadins in unsonicated and sonicated 4 mM HAc solutions are not different from each other. However, the partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, of crude gliadins in sonicated 4 mM HAc solutions is larger than that in unsonicated

solutions. Since lipids dissociate from gliadins in acidic solutions (McCann *et al*., 2009), the increase in $\bar{\beta}_S^o$ of crude gliadins in sonicated 4 mM HAc is not because of the formation of protein-lipid complexes as in ethanolic solutions.

It has been introduced that the net charges of the protein molecules are increased in dilute acid and therefore crude gliadins are soluble in 4 mM HAc (Damodaran, 1996; McCann *et al*., 2009). Chalikian *et al*. (1995) introduced the idea that globular proteins have compact intermediate (CI) molecular states with large values of partial specific adiabatic compressibility coefficient in acidic solutions (*Section 2.1.3.3*). Although in unsonicated 4 mM HAc solutions, gliadins are not supposed to have CI states based on the small values of $\bar{\beta}_S^o$, crude gliadins in sonicated dilute acidic solutions might have CI molecular states, which accounts for the loose intrinsic structures of gliadins as indicated by increased value of $\bar{\beta}_S^o$. The sonication treatment might contribute to the transition from native (N) state to the compact intermediate (CI) state of proteins in solutions and increase their surface activity (Güzey *et al*., 2006).

Results of apparent specific volume, $\bar{v}$, and the apparent specific adiabatic compressibility, $\bar{K}_S$, of crude gliadins in 4 mM HAc with different treatments (without and with sonication) are shown in Figure 5.12 and Figure 5.13.
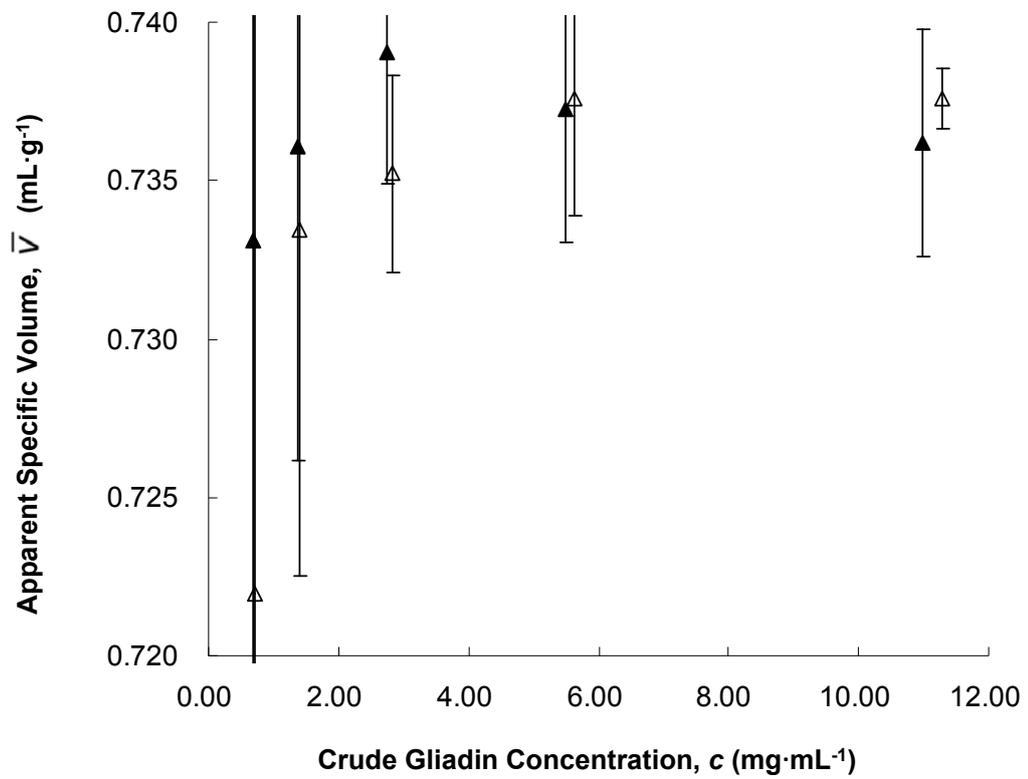
Figure 5.12 Apparent specific volume, $\bar{v}$, of crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (Δ).
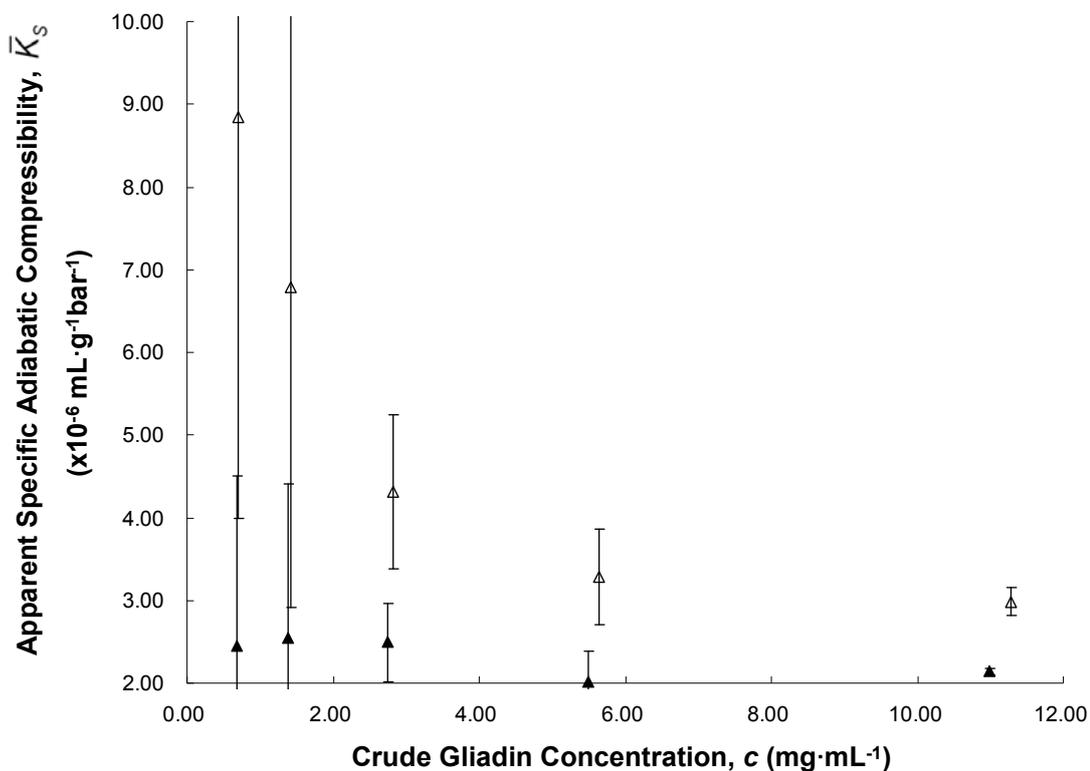
Figure 5.13 Apparent specific adiabatic compressibility, $\bar{K}_S$, of crude gliadins in 4 mM HAc (▲) and sonicated ones in 4 mM HAc (△).

As shown in Figure 5.12 the apparent specific volumes, $\bar{v}$, of crude gliadins in 4 mM HAc without and with sonication treatment are not different from each other at each concentration. A potential effect of dilution on decreasing the values of $\bar{v}$ of crude gliadins in both (without and with sonication treatment) 4 mM HAc solutions is observed, which is consistent with the results of Krejci and Svedberg (1935), who found the apparent specific volume of gliadins dropped from 0.736 to 0.712 mL·g^{-1} as the gliadin concentration of 8 mM HCl – 8 mM KCl aqueous solutions was dropped from 1.96% to 0.25%. However, the apparent specific

volume, $\bar{v}$, provides insufficient information on molecular states of proteins (Chalikian *et al*., 1995; Chalikian, 2001), and the results of $\bar{K}_S$ are discussed.

From Figure 5.13 the values of $\bar{K}_S$ of crude gliadins in unsonicated 4 mM HAc solutions are independent of concentrations of crude gliadins. Conversely, the values of $\bar{K}_S$ of crude gliadins in sonicated 4 mM HAc solutions increase as the solutions are diluted. Beside, the values of $\bar{K}_S$ of crude gliadins in sonicated 4 mM HAc solutions are consistently larger than those of unsonicated samples. It has been speculated that sonication treatment contributes to the transition from N to CI protein molecular states. According to Chalikian *et al*. (1995), partial specific adiabatic compressibility increases as the degree of transition from N to CI state increases. It can be speculated that dilution of crude gliadins in sonicated 4 mM HAc solutions contributes to this N-CI transition of gliadins. However, this conclusion of dilution effects on crude gliadins in sonicated 4 mM HAc would require substantiation.

5.6. Conclusions of Solvent Analyses

In 70% (v/v) EtOH, crude gliadins exist as complexes formed by gliadins and LMW-gluteinins and lipids according to the results of the present experiments and previous studies (Békés *et al*., 1983a; Békés *et al*., 1983b; Békés *et al*., 1992; McCann *et al*., 2009). These protein-lipid complexes result in large partial specific volume, $\bar{v}^o$ (0.747 mL·g^{-1}), and larger partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ (10.27 ×10^{-6} bar^{-1}) for ethanolic soluble crude gliadins, because

of an extra solvent inaccessible interior of the complex, which increases both the intrinsic volume and the intrinsic compressibility. The protein-lipid interactions in ethanol solutions are not thought to be disturbed by sonication treatment. The protein-lipid complexes might be destroyed by dilution in ethanolic solutions and the gliadin proteins might be denatured by dilution. However, more work needs to be done for confirmation.

In 4 mM HAc, unsonicated crude gliadins have good repeatability according to small errors in the results of $\bar{v}^o$ and $\bar{\beta}_S^o$. This probably is because of smaller effect of solvent evaporation on the density and the ultrasound velocity values. There is no protein-lipid complex existing in dilute acidic solutions according to the results of $\bar{v}^o$ (0.736 mL·g^{-1}), and $\bar{\beta}_S^o$ (3.72 ×10^{-6} bar^{-1}) of crude gliadins in 4 mM HAc and according to previous studies (McCann *et al.*, 2009).

Sonication treatment tends to weaken the intra-molecular hydrophobic interactions that stabilize the gliadins and leads to an N-to-Cl transition of gliadins. Therefore, sonication increases the values of $\bar{K}_S$ and $\bar{\beta}_S^o$ of crude gliadins in both solutions (70% EtOH and 4 mM HAc), especially for the crude gliadins in 4 mM HAc. However, more studies are needed to confirm the effects of sonication and dilution on the proteins.

Based on the knowledge that proteins with large values of $\bar{\beta}_S^o$ (> 4 ×10^{-6} bar^{-1}) have good functionalities (e.g., interfacial properties) as introduced in *Section 2.1.3.4*, and the amount of complexes formed by ethanol soluble LMW-glutenins and lipids have positive contributions to flour suitability for breadmaking (Békés *et al.*, 1992), it is possible to establish a relationship between the compressibility

properties of gliadins, e.g., $\bar{\beta}_s^o$ and $\bar{K}_s$, and the contribution of gliadins to flour baking suitability. Based on the present experimental results, complexes formed by both ethanol soluble LMW-glutenins and gliadins and lipids might have positive contributions to flour baking suitability, consistent with the conclusion of Békés *et al.* (1992).

6. Flour Analyses Results and Discussion

The flour analyses involve the comparisons of density and ultrasound velocity values of crude gliadins extracted from different flours. The purpose of these analyses is to evaluate whether there are differences in the properties of crude gliadins from different flours. Crude gliadins were extracted from flour samples of two different wheat classes: SWS and CWES (*Section 3.1.1*). The only solvent used for these analyses was 4 mM HAc because the solvent properties are less affected by evaporation than aqueous ethanol, and the potential effects of dilution on crude gliadins are not strong (*Section 5.6*). The density, ultrasound velocity and ultrasound attenuation values of acidic soluble crude gliadins from the two different flours were determined and compared with each other and with the values of acidic soluble crude gliadins from CWRS flours which were determined in the solvent analyses.

6.1. Protein Content of Flours, Crude Gliadins and Soluble Crude Gliadins

The protein contents of different flours (1 SWS, 1 CWRS, and 1 CWES), lyophilized crude gliadin powders and acidic soluble crude gliadins in the most concentrated solutions were determined by micro-Kjeldahl method (*Section 3.2.5*). The results (Table 6.1) show the protein contents of lyophilized crude gliadin powders from flours (3rd column) and the soluble crude gliadins in 4 mM HAc (4th column) are related to those of the flours (2nd column).

Table 6.1 Protein content of different flours, lyophilized crude gliadin powders, and acidic soluble crude gliadins in 4 mM HAc solutions.

Flours source	Protein content (%)		
	Flours	Lyophilized crude gliadin powders	Acidic soluble crude gliadins
SWS	9.2 ± 0.1[1]	60 ± 1	66.3 ± 1.2
CWRS	13.6 ± 0.1	73 ± 2	79.1 ± 0.6
CWES	12.0 ± 0.2	63 ± 1	68.1 ± 0.2

1. mean $\pm$ standard deviation (obtained from 3 replicates)

6.2. Density and Ultrasound Results

The specific density, specific ultrasound velocity, and specific ultrasound attenuation, calculated as $\rho_{sp} = \rho/\rho_o - 1$, $u_{sp} = u/u_o - 1$ and

$$a_{sp} = \frac{a}{a_o} - 1 = \frac{\left(a/f^2\right)}{\left(a/f^2\right)_o} - 1$$ (*Section 5.2*) for different soluble crude gliadins in 4 mM HAc, are shown in Figure 6.1 to Figure 6.3.
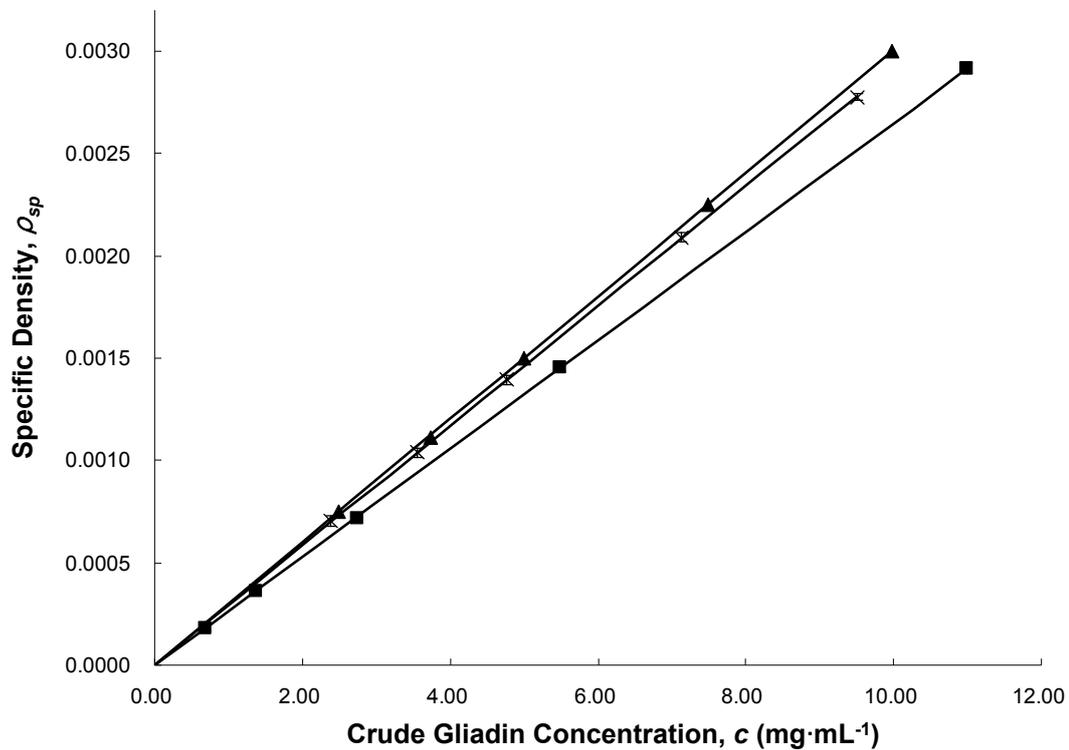
Figure 6.1 Specific density, ρ_{sp}, of serial dilutions of crude gliadins extracted from 1 SWS (▲), 1 CWRS (■) and 1 CWES (X).

Figure 6.2 Specific ultrasonic velocity, u_{sp}, of serial dilutions of crude gliadins extracted from 1 SWS (▲), 1 CWRS (■) and 1 CWES (X).
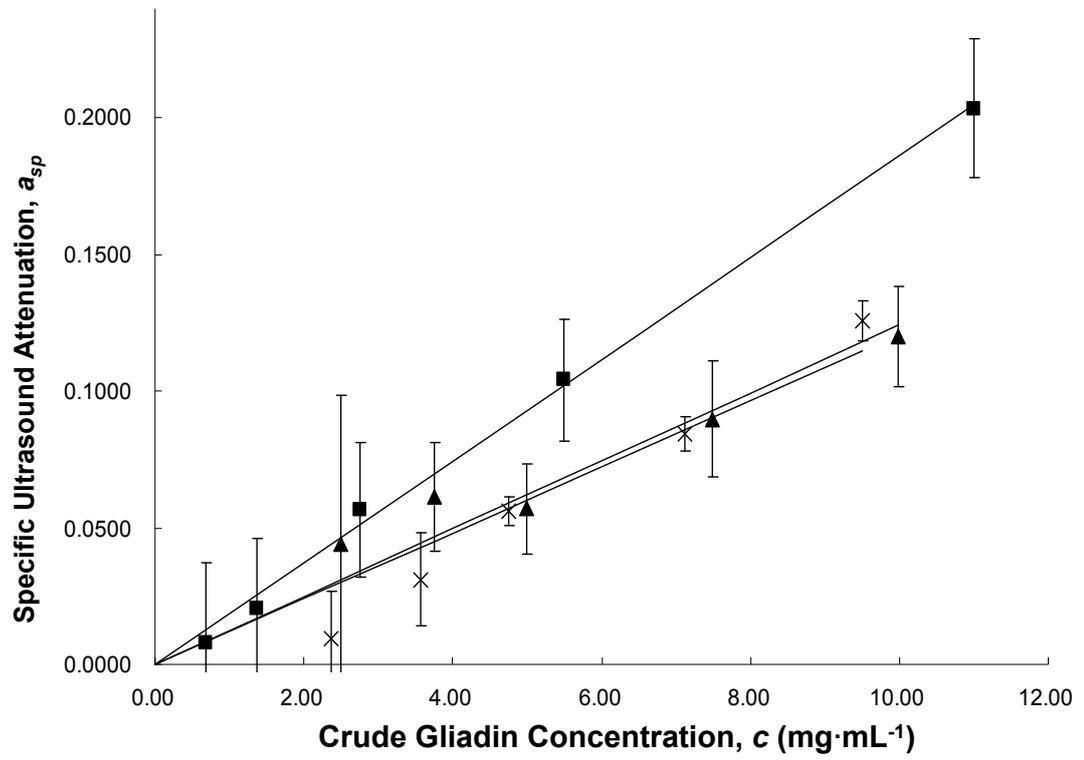
Figure 6.3 Specific ultrasonic attenuation, a_{sp}, of serial dilutions of crude gliadins extracted from 1 SWS (▲), 1 CWRS (■) and 1 CWES (X).

According to the definition of specific quantities, the intercepts of the regression lines of ρ_{sp}, u_{sp}, and a_{sp}, in the figures are set to be 0. The regression lines (with intercept at 0) have good R^2 values (> 0.94), especially those of ρ_{sp} and u_{sp} ($R^2 > 0.99$), indicating that there is no aggregation or variation of the soluble crude gliadins and the solutions can be treated as ideal (Pavlovskaya *et al.*, 1992). Besides, the error bar attached to each point in Figure 6.1 and Figure 6.2 is very small, indicating good repeatability. However, the error bars of specific ultrasound attenuation are large.

The concentration dependences of specific density, specific ultrasound velocity and specific ultrasound attenuation of different crude gliadins in solutions, numerically equaling the slopes ($[\rho]_{sp}$, $[u]_{sp}$, and $[a]_{sp}$) of the plots of ρ_{sp}, u_{sp}, and a_{sp} against concentration c, as well as the R^2 value of each regression line, are shown in Table 6.2 to Table 6.4.

Table 6.2 Concentration dependence of specific density, $[\rho]_{sp}$, of serial dilutions of crude gliadins from different flours.

Flour source	$[\rho]_{sp}$ ×10^{-4} (mL·mg^{-1})	R^2
SWS	3.005	1.000
CWRS	2.649	1.000
CWES	2.926	1.000

Table 6.3 Concentration dependence of specific ultrasound velocity, $[u]_{sp}$, of serial dilutions of crude gliadins from different flours.

Flour source	$[u]_{sp}$ $\times 10^{-4}$ (mL·mg^{-1})	R^2
SWS	2.072	1.000
CWRS	2.138	1.000
CWES	2.078	1.000

Table 6.4 Concentration dependence of specific ultrasound attenuation, $[a]_{sp}$, of serial dilutions of crude gliadins from different flours.

Flour source	$[a]_{sp}$ (mL·mg^{-1})	R^2
SWS	0.0121	0.944
CWRS	0.0186	0.997
CWES	0.0124	0.947

Separation of the three curves in all three figures (from Figure 6.1 to Figure 6.3) means that there are differences in the density and ultrasonic properties of crude gliadins from different flours. It can be seen in the above figures that crude gliadins from 1 CWRS have different effects on density and ultrasonic properties

of the acidic solutions compared with those from 1 CWES and 1 SWS flours, which are evident from Table 6.2 to Table 6.4. The loaf volumes of these three classified flours are reported as CWES > CWRS > SWS (Sapirstein and Suchy, 1999; Zghal *et al.*, 2001). Thus, the density and ultrasonic properties of soluble crude gliadins in acidic solutions seem not to be related to the baking suitability of source flours but to the protein contents of the acidic soluble crude gliadins, as well as the protein contents of the source flours. From Table 6.2 and Table 6.3, the proteins in acidic soluble crude gliadins have less increasing effects on density but more increasing effects on ultrasound velocity of crude gliadin solutions than non-protein components.

6.3. Discussions on Flour Analyses

6.3.1. Relationship between $[\rho]_{sp}$, $[u]_{sp}$ and $\bar{v}^o$, $\bar{\beta}_S^o$ of Proteins

Concentration increments of density, $[\rho]$ and ultrasound velocity, $[u]$ have been defined by Sarvazyan (1991) as $[\rho] = \lim\limits_{c \to 0} \dfrac{\rho - \rho_o}{\rho_o c}$ and $[u] = \lim\limits_{c \to 0} \dfrac{u - u_o}{u_o c}$ (Equations 17 and 18 in *Section 2.1.2.3*). Mathematically, the expressions $\dfrac{\rho - \rho_o}{\rho_o}$ and $\dfrac{u - u_o}{u_o}$ represent the specific density, ρ_{sp} and the specific ultrasonic velocity, u_{sp}, which have good linearity with the concentrations of soluble crude gliadins in 4 mM HAc. Thus, the values of $\dfrac{\rho - \rho_o}{\rho_o c}$ and $\dfrac{u - u_o}{u_o c}$ are constant and

equal to $[\rho]_{sp}$ and $[u]_{sp}$, respectively, and $[\rho]_{sp}$ and $[u]_{sp}$ can be used as good estimates for $[\rho]$ and $[u]$.

The expression for apparent specific volume, $\bar{v} = \dfrac{1-(\rho-c)/\rho_o}{c}$ (Equation 2 in *Section 2.1.1.1*) can be rewritten as $\bar{v} = \dfrac{1}{\rho_o} - \dfrac{\rho-\rho_o}{\rho_o c}$ where $\dfrac{\rho-\rho_o}{\rho_o c}$ is observed to be constant and equal to $[\rho]_{sp}$. So the equation $\bar{v} = 1/\rho_o - [\rho]_{sp}$ is obtained. Since $[\rho]_{sp}$ is independent of solute concentrations for ideal solutions, the partial specific volume, $\bar{v}^o$, is estimated to be equal to the apparent specific volume, $\bar{v}$ (Chalikian *et al.*, 1996).

Additionally, from $[\beta_S] = -2[u] - [\rho]$ and $\bar{K}_S = \beta_{So}(\bar{v} + [\beta_S])$ as introduced by Sarvazyan (1991) (Equations 20 and 21 in *Section 2.1.2.3*) and the estimations $[\rho] = [\rho]_{sp}$, $[u] = [u]_{sp}$ and $\bar{v} = 1/\rho_o - [\rho]_{sp}$, the expression, $\bar{K}_S = \beta_{So} \times \left(1/\rho_o - 2[\rho]_{sp} - 2[u]_{sp}\right)$, for apparent specific adiabatic compressibility is obtained. Both $[\rho]_{sp}$ and $[u]_{sp}$ are independent of solute concentrations for ideal solutions (Pavlovskaya *et al.*, 1992), so the partial specific adiabatic compressibility, $\bar{K}_S^o$, is estimated to be equal to the apparent specific adiabatic compressibility, $\bar{K}_S$ (Chalikian *et al.*, 1996).

From the general relationship between partial specific adiabatic compressibility and its coefficient $\bar{\beta}_S^o = \dfrac{\bar{K}_S^o}{\bar{v}^o}$ (Equation 13 in *Section 2.1.2.3*) and

the estimates of $\bar{v}^o$ and $\bar{K}_S^o$, the partial specific adiabatic compressibility coefficient is estimated as

$$\bar{\beta}_S^o = \beta_{So} \times \left(2 - \frac{2[u]_{sp} + 1/\rho_o}{1/\rho_o - [\rho]_{sp}} \right)$$

6.3.2. Volumetric and Compressible Properties of Different Crude Gliadins

From the equations discussed in *Section 6.3.1*, when the values of $[\rho]_{sp}$ are in the range $0 < [\rho]_{sp} < 1/\rho_o$ as observed in the experiment, the following relationships are established: $\bar{v}^o \propto \left(-[\rho]_{sp} \right)$ and $\bar{\beta}_S^o \propto [u]_{sp}/[\rho]_{sp}$. In terms of these relationships, $\bar{v}^o$ and $\bar{\beta}_S^o$ of crude gliadins from different flours can be compared based on the values of $-[\rho]_{sp}$ and $[u]_{sp}/[\rho]_{sp}$ (Table 6.5).

Table 6.5 Values of $-[\rho]_{sp}$ and $[u]_{sp}/[\rho]_{sp}$ for acidic solutions of crude gliadins from different flours.

Flour source	$-[\rho]_{sp} \times 10^{-4}$ (mL·mg^{-1})	$[u]_{sp}/[\rho]_{sp}$
SWS	-3.005	0.688
CWRS	-2.649	0.808
CWES	-2.926	0.710

From the results shown in Table 6.5, it is estimated that in 4 mM HAc, the crude gliadins extracted from 1 CWRS flours have the highest values of $\bar{v}^o$ and $\bar{\beta}_S^o$, as indicated by high values of $\left(-[\rho]_{sp}\right)$ and $[u]_{sp}/[\rho]_{sp}$, and those extracted from 1 SWS flours have the smallest values of $\bar{v}^o$ and $\bar{\beta}_S^o$.

Correlation coefficients for relationships between protein contents of flours (FP), lyophilized crude gliadin powders (LP) and acidic soluble crude gliadins (AP) and the results of $[\rho]_{sp}$, $[u]_{sp}$, and $[u]_{sp}/[\rho]_{sp}$ are summarized in Table 6.6.

Table 6.6 Correlations between protein contents of different materials (FP, LP and AP) and density and ultrasound velocity behavior ($[\rho]_{sp}$, $[u]_{sp}$, and $[u]_{sp}/[\rho]_{sp}$) of acidic soluble crude gliadins.

	$[\rho]_{sp}$	$[u]_{sp}$	$[u]_{sp}/[\rho]_{sp}$
FP[a]	-0.889	0.821	0.870
LP[b]	-1.000**	0.990	0.999*
AP[c]	-0.997	0.999*	0.999*

* $P < 0.05$; ** $P < 0.01$

a. FP: protein contents of flours

b. LP: protein contents of lyophilized crude gliadin powders

c. AP: protein contents of acidic soluble crude gliadins

The correlation coefficient values shown in Table 6.6 are quite high but some of them are not significant at $P < 0.05$, a fact that is likely because of the small sample size ($n = 3$). Significant correlations ($P < 0.05$) are found between AP and $[u]_{sp}$, AP and $[u]_{sp}/[\rho]_{sp}$, LP and $[\rho]_{sp}$, and LP and $[u]_{sp}/[\rho]_{sp}$. It is reasonable to expect strong correlations between protein content of acidic soluble crude gliadins (AP) and density and ultrasound behavior ($[\rho]_{sp}$, $[u]_{sp}$, and $[u]_{sp}/[\rho]_{sp}$) of acidic soluble crude gliadins. Results of $[\rho]_{sp}$ have a more significant ($P = 0.0059$) correlation with protein content of lyophilized crude gliadin powders (LP) than with AP ($P = 0.0526$). However, as discussed, small sample size provides insufficient confidence on the results of significance.

From Table 6.5 and Table 6.6, values of $\bar{v}^o$ and $\bar{\beta}_S^o$ seem to be related to the protein contents of different soluble crude gliadins in the solutions. Proteins have positive contributions to $\bar{v}^o$ and $\bar{\beta}_S^o$ of soluble crude gliadins in 4 mM HAc solutions while non-protein components (lipids and small amount of carbohydrates) have negative contributions. The reason might be that the latter molecules have no solvent inaccessible void space which is abundant in globular protein molecules and contributes positively to $\bar{v}^o$ and $\bar{\beta}_S^o$ of the molecules (Chalikian *et al*., 1996 and related references in *Section 2.1.3.2.1*).

6.4. Conclusions of Flour Analyses

In dilute acidic solutions of gliadin proteins the partial specific parameters ($\bar{v}^o$, $\bar{K}_S^o$ and $\bar{\beta}_S^o$) can be well estimated by apparent specific ones ($\bar{v}$, $\bar{K}_S$ and $\bar{\beta}_S$) because there is no concentration dependence of apparent specific quantities. Additionally, the values of $[\rho]_{sp}$ and $[u]_{sp}$, which shows the ability of soluble proteins to cause relative fractional changes in the density and ultrasonic velocity, can be used for estimations for the values of $\bar{v}$, $\bar{K}_S$ and $\bar{\beta}_S$, as well as $\bar{v}^o$, $\bar{K}_S^o$ and $\bar{\beta}_S^o$.

There is no evidence showing that gliadins differ with different wheat flours and cause different values for the $\bar{v}^o$ and $\bar{\beta}_S^o$ of crude gliadins. The values of $\bar{v}^o$ and $\bar{\beta}_S^o$ of crude gliadins in 4 mM HAc seem to have no relationship with breadmaking suitability of their source flours, which is consistent with the finding of Sapirstein and Fu (1996), who stated that gliadins were not related to baking qualities of different flour cultivars.

7. Conclusion and Recommendation

7.1. Conclusion

In this thesis, the partial specific volume, $\overline{v}^o$, and the partial specific adiabatic compressibility coefficient, $\overline{\beta}_S^o$, for crude gliadins in different solvents were reported. From these thermodynamic parameters, the effects of solvents and sonication treatment on gliadin structure and conformation were discussed.

In the preliminary experiments, the ultrasound velocity values of crude gliadins in ethanolic solutions exhibited non-linearity with solution concentrations. The reason might be the possible contamination of gliadin extracts from salt and the possible effects of salts on the protein structures in Osborne fractionation (Arrhenius, 1937; Clements, 1973; Fu *et al*., 1996). Besides, the ultrasound velocity of the 70% (v/v) ethanol used is about 5 m·s⁻¹ different from the reference value (D'Arrigo and Paparelli, 1988). The complicated composition of the solvents (ethanol-63% v/v, methanol-3.5% v/v, isopropanol-3.5% v/v) is supposed to account for this difference. As a result, fresh 70% (v/v) aqueous ethanol solutions made of 95% (v/v) ethanol and water were selected to extract the crude gliadins from wheat flours and to be used as a solvent of the crude gliadins. Thus, the possible effects of salts on gliadin proteins during extraction were excluded and the solvent would have similar physical properties to those reported in previous studies.

The density, the ultrasound velocity and the ultrasound attenuation were measured for ethanolic extracted crude gliadins in 70% (v/v) EtOH and 4 mM HAc

solutions, without or with sonication treatment. The three physical properties exhibited good linearity with crude gliadin concentration in both 70% (v/v) EtOH and 4 mM HAc, regardless of whether sonication treatment was applied or not, indicating that there was no interaction between the solute molecules, and the solutions could be treated as ideal (Pavlovskaya *et al.*, 1992). Accordingly, further studies of density and ultrasound velocity properties and the partial specific adiabatic compressibility coefficients for crude gliadins in different solvents were performed for the solvent and flour analyses.

For the solvent analyses, solvent and sonication effects on crude gliadins were discussed in terms of the values of the partial specific volume, $\bar{v}^o$, and the partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$, for crude gliadins. The ethanolic soluble crude gliadins had larger partial specific volume, $\bar{v}^o$ (0.747 mL·g^{-1}), and larger partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ (10.27 ×10^{-6} bar^{-1}) than those for acidic soluble crude gliadins ($\bar{v}^o = 0.736$ mL·g^{-1}; $\bar{\beta}_S^o = 3.72 \times 10^{-6}$ bar^{-1}). These results were thought to be evidence for the existence of complexes formed by gliadins and LMW-glutenins and lipids in ethanol solutions. It had been reported that LMW-glutenins and gliadins in ethanol soluble gliadin-rich fractions form complexes with lipid in ethanol solutions (Békés *et al.*, 1983a; Békés *et al.*, 1983b; Békés *et al.*, 1992; McCann *et al.*, 2009), and these complexes were disrupted and absent in acidic solutions (McCann *et al.*, 2009). Based on the present results, ethanolic soluble crude gliadins had larger values of $\bar{v}^o$ and $\bar{\beta}_S^o$ than acidic soluble ones because of an extra solvent inaccessible interior of the protein-lipid complexes in

crude gliadins, which increases both the intrinsic volume and the intrinsic compressibility.

Sonication treatment was observed to have the potential to increase the values of apparent specific adiabatic compressibility, $\bar{K}_S$, and the partial specific adiabatic compressibility coefficient, $\bar{\beta}_S^o$ for crude gliadins in both solvents (70% EtOH and 4 mM HAc), especially for the crude gliadins in 4 mM HAc. Based on these observations, sonication was supposed to weaken the intra-molecular hydrophobic interactions that stabilize the gliadins and leads to an N-to-CI transition of gliadin proteins, which was consistent with the results from Güzey *et al*. (2006) who proposed sonication favored protein unfolding but did not denature the proteins.

It was also found that dilution had effects on the structures and conformations of crude gliadins, especially in sonicated solutions. Dilution might favor the denaturation of gliadin proteins in ethanolic solutions and the effects were enhanced by sonication treatment. However, considering the experimental errors for the results, more studies are needed to confirm the effects of sonication and dilution on the proteins.

In the flour analyses, crude gliadins were extracted from wheat flours of different classes and dissolved in 4 mM HAc solutions. The density and ultrasound velocity properties were discussed. In this part of the thesis, the partial specific parameters ($\bar{v}^o$, $\bar{K}_S^o$ and $\bar{\beta}_S^o$) were well estimated by apparent specific ones ($\bar{v}$, $\bar{K}_S$ and $\bar{\beta}_S$) in dilute acidic solutions of gliadin proteins because there is no concentration dependence of apparent specific quantities. Additionally, the

values of $[\rho]_{sp}$ and $[u]_{sp}$, which showed the ability of soluble proteins to cause relative fractional changes in the density and ultrasonic velocity, were used for estimations of the values of $\bar{v}$, $\bar{K}_S$ and $\bar{\beta}_S$, as well as $\bar{v}^o$, $\bar{K}_S^o$ and $\bar{\beta}_S^o$. There was no evidence showing that gliadins change with different wheat flours and cause different values for the $\bar{v}^o$ and $\bar{\beta}_S^o$ of crude gliadins. The values of $\bar{v}^o$ and $\bar{\beta}_S^o$ of crude gliadins in 4 mM HAc seem to have no relationship with breadmaking suitability of their source flours. However, they were found to be related to the protein contents of the solutes (as well as the protein contents of the flours) and proteins had positive contributions to $\bar{v}^o$ and $\bar{\beta}_S^o$ of acidic soluble crude gliadins and non-protein components had negative contributions.

7.2. Recommendation

One objective of this thesis was to determine solvent effects on the properties of gliadins. The impurity of gliadins and the possible aggregation of gliadins during lyophilization that cause the freeze dried crude gliadins to be not completely soluble in 70% (v/v) EtOH are problems in analyses of the results. Additionally, the dilution and sonication effects on gliadin properties remain to be confirmed. For further studies, it is recommended that pure gliadins should be used for research. The gliadins should be extracted and purified in mild conditions and used for determination of the solvent effects on protein conformation. For understanding the conformations of pure gliadins in different solvents, the partial specific adiabatic compressibility coefficient of pure gliadins in 70% (v/v) EtOH should be

determined and used as a baseline for comparison with the partial specific adiabatic compressibility coefficients of pure gliadins in dilute acid (e.g., HAc), and aqueous ethanol with salts.

Another objective of this project was to establish a relationship between the compressibility properties and the gliadin functionalities in breadmaking suitability of the flours. Based on the knowledge that proteins with large values of $\bar{\beta}_S^o$ (> 4 ×10^{-6} bar^{-1}) have good functionalities (e.g., interfacial properties) as introduced in *Section 2.1.3.4*, and the present experimental results, protein-lipid complexes formed by ethanol soluble LMW-glutenins and gliadins and lipids might have positive contributions to flour baking suitability, consistent with the conclusion of Békés *et al.* (1992). For further research, protein-lipid complexes from wheat flours of different classes might be studied. The purpose of such a study would be to determine if ethanolic soluble crude gliadins relate to the flour baking quality to see if protein-lipid complexes are one of the reasons causing the different baking suitability of flours.

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