CHAPTER 3

PHYSICOCHEMICAL PROPERTIES OF HEAT AND HIGH PRESSURE INDUCED TOFU GELS

3.1 Materials

All of the chemicals used were of analytical grade from commercial services and stored under appropriate conditions.

Soybeans (*Glycine max*) were purchased from Morning Foods Ltd., Crewe, UK. (MF-soybean). The dried beans were stored at 4°C until required.

3.2 Methods

3.2.1 Preparation of tofu samples

Tofu samples used in these experiments were filled (packed) tofus prepared from soymilk using various coagulants without the elimination of the whey. The coagulants were glucono-δ-lactone (GDL, $C_6H_{10}O_6$); calcium sulphate dihydrate ($CaSO_4.2H_2O$) and calcium chloride dihydrate ($CaCl_2.2H_2O$) all of which were purchased from Sigma. The tofu samples were prepared by two different physical treatments, heat induced (Ht) coagulation and high pressure induced (HP) coagulation.

3.2.1.1 Soymilk preparation

Soymilk was prepared from the whole soybean. Washed soybean was soaked overnight at room temperature (20-25°C) in 10 times its dry seed weight of deionised water. After draining off excess water, the swollen bean was ground with five times its dry weight of deionised water using a grinder/extractor (Central Industrial Supply Ltd, Bangkok, Thailand). The resultant slurry was then filtered through a filter cloth to remove any solid pulp (okara) then subsequently centrifuged at 1200 g and 10°C for 5 min (Sorvall® RC 5 B plus, DuPont, USA). The soymilk was collected from the

supernatant and kept at 4°C for further processing. The resulting soymilk was used to produce tofu within one week.

3.2.1.2 Heat induced tofu preparation

Soymilk was preheated in a boiling water bath to 97-100°C for 7 min with regular stirring. It was then cooled in an ice bath to room temperature (approximately 7 min). Subsequently 3 ml of the chosen coagulant solution was added to 100 ml of the preheated soymilk in a 250 ml beaker (Pyrex) at room temperature to make the final concentration of coagulant as required. The mixture was subsequently uniformly mixed using an overhead stirrer (propeller of 25 mm diameter, at 250 rpm for 20 sec) then heated in water bath at 70°C for 60 min (Shen *et al*., 1991; Obata and Matsuura, 1993). After which the warm sample was immediately cooled in an ice bath to room temperature and held at room temperature (20-25°C) for 60 min prior to storage at 4°C.

3.2.1.3 High pressure induced tofu preparation

High pressure (HP) induced tofu gels were prepared from raw soymilk with a suitable coagulant. The coagulant solution was added to the raw soymilk and uniformly mixed using the same methods used to prepare Ht tofu samples. The mixture then was sealed in a polyethylene bag (Cryovac Ltd, UK) and subjected to a high pressure using a 'Food lab' high pressure rig (Stansted Fluid Power, Stansted, Essex, UK). The pressurization rate was found to be about 250 MPa/min, when using a mixture of castor oil and ethanol (20:80) as pressure transmission media. After treatment samples were aged at room temperature (20-25°C) for 60 min prior to storage at 4°C. There was no evidence of ingress of transmission oil into sample.

3.2.2 Chemical analysis of soybean, soymilk and tofu

Soybeans were analysed for their seed size (by weighing of 100 beans), moisture content (Hot air oven method; AOAC, 2000), total protein (Kjeldahl method; AOAC, 2000), total fat (Soxhlet method; AOAC, 2000), inorganic ash (AOAC, 2000), and total carbohydrate (by subtraction; % total carbohydrate = 100 - % moisture

content - % fat - % protein - % ash). The swollen bean mass was determined from the weight of the overnight soaked beans, which were then drained and weighted and compared to the dried soybean material.

Soymilks were examined for pH (using an Orion pH meter 420), total solids, total moisture content, total protein levels, total extractable fat, inorganic ash, and total carbohydrate. Tofu samples were examined for their pH value, moisture content and total protein content.

3.2.3 Determination activity of trypsin inhibitors

Activity of trypsin inhibitors (TI) in soybean and soymilks were determined based on the method proposed by Hamerstrand *et al*. (1981), Kakade *et al*. (1974) and Prinyawiwatkul *et al.* (1996). One gram of sample was mixed with 50 ml of 0.01 N NaOH and adjusted pH to 8.4-10.0 by 0.1-1N HCl. After stirring for 3 hr, the mixture was filtered through filter paper (Whatman No. 3) and the filtrate was diluted so that 2 ml of the diluted filtrate would inhibit 40-60% activity of trypsin inhibitors in an equal volume of trypsin standard solution (EC 3.4.21.4, from Bovine pancreas, salt-free, Sigma).

The mixture of the diluted filtrate and trypsin standard solution was allowed to react at 37±1°C for 10 min in a water bath, subsequently mixed with N α-benzolyl-DL-arginine-p-nitroanilide substrate (BAPA, Sigma) which was prewarmed to 37°C. The mixture was allowed to react for another 10 min. at 37±1°C, then this reaction was terminated by adding 1 ml of 30% (v/v) acetic acid. The extracted sample and trypsin standard solution were measured for their absorbance at 410 nm to determine *p*-nitroaniline released from the substrate as a result of trypsin action (Kakade, 1969).

Absorbance reading from each determination was subtracted from the absorbance of trypsin standard. Each treatment was determined for four replications. Since 1 μg of pure trypsin has an activity equivalent to 0.019 absorbance units (Kakade *et al*., 1969), the trypsin inhibitor activity was determined using the following

relationship (Hamerstrand *et al*., 1981):

TI, mg/g of sample = (differential absorbance * dilution factor)/ (0.019*1,000)

3.2.4 Determination of surface hydrophobicity

Surface hydrophobicity of protein of raw, heated (Ht) and pressurised (HP; 400 MPa 20°C 10 min.) soymilk were determined using a hydrophobic probe of 1-anilino-8-napthalene sulfonate (ANS, Sigma) (Hayakawa and Nakai, 1985; Wu, *et al*., 1998). The sample was diluted with 0.01 M phosphate buffer (pH 7.0) to a protein concentration range from 0.00056 to 0.015%. A twenty micro litre of ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 4 ml of diluted soymilk and stood for two hours at room temperature (20°C). Consequently the diluted soymilk was determined by spectrofluorometer 1100 (LS-5 Luminescene Spectrometer, Perkin Elmer) for fluorescence intensity (FI), using excitation and emission wavelength of 390 and 470 nm, respectively. The measuring system was standardised with 10 µl of ANS in 5 ml methanol to 80% of reading full scale. The slope of the plots of the FI versus percentage of protein concentration in soymilk was calculated by least square linear regression and used as the surface hydrophobicity (S_0). All determination was performed in triplicate.

3.2.5 Rheological measurements on tofu

A controlled stress rheometer (Rheo-Tech International Ltd, Royston, England) was used to measure the dynamic viscoelastic properties of the various samples. Measuring geometry used was a 20 mm diameter parallel plate with a gap width of 2 mm. Samples were loaded onto the rheometer and allowed to equilibrate to the measuring temperature (25°C, about 3 min). Excess sample was trimmed off carefully with a razor blade. A thin layer of low viscosity silicone lubricant (less than 1 cp) was applied to the exposed free edges to prevent evaporation of water. Subsequent examination of the samples after testing showed no evidence of any ingress of this oil into the sample.

The linear viscoelastic region of the various samples was determined by means of a series of oscillation stress sweeps (0.5 to 10 Pa at a frequency of 1 Hz) as shown in Figure 3.1 and 3.2.

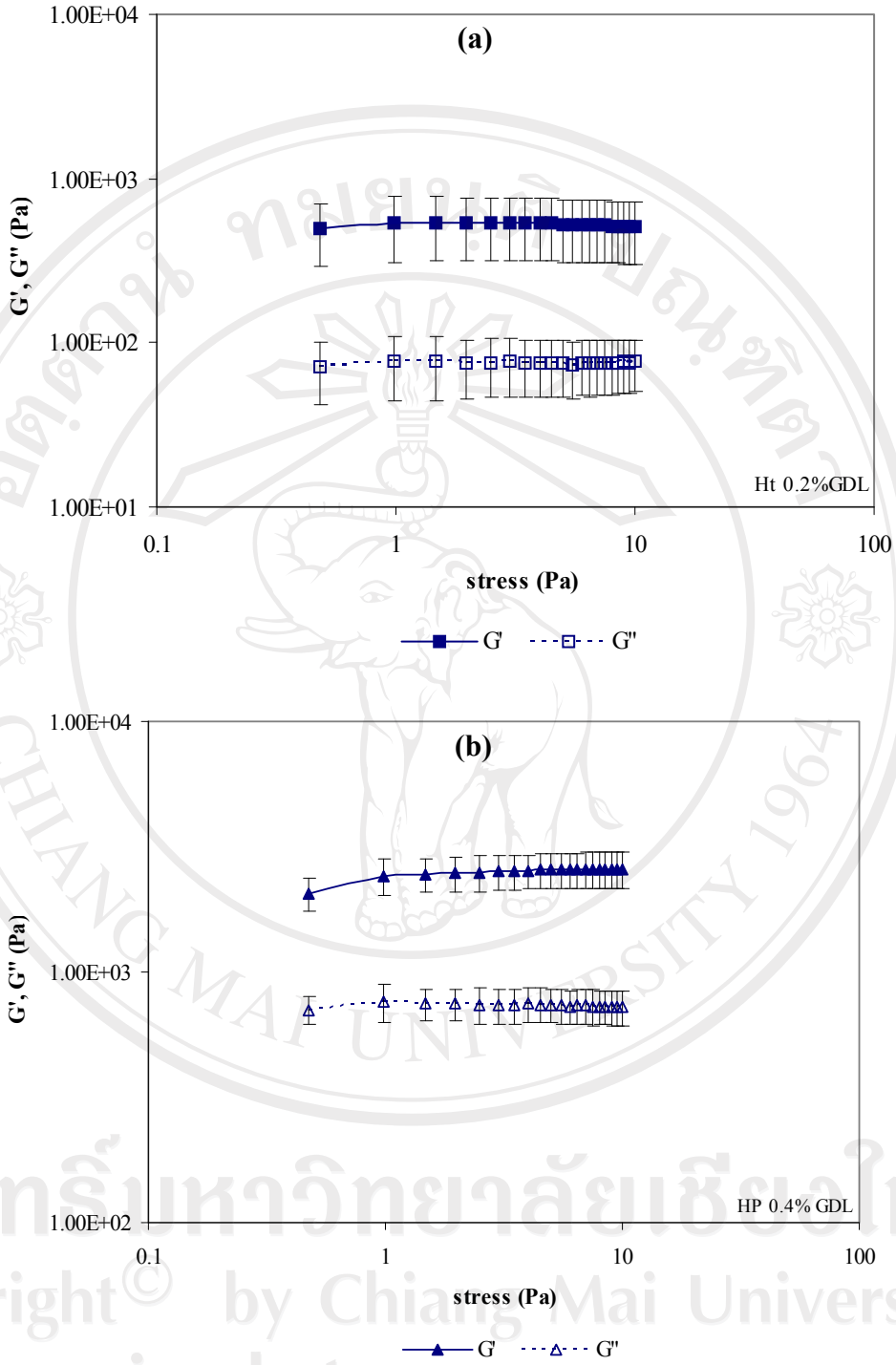

Figure 3.1 The storage (G') and loss (G'') moduli of GDL tofu gels as a function of the stress amplitude (0.5 to 10 Pa) at a frequency of 1 Hz (mean, n = 6) for: (a) 0.2% w/v GDL heat induced tofu gel; (b) 0.4% w/v GDL high pressure (400 MPa and 20°C for 10 min) induced tofu gel.

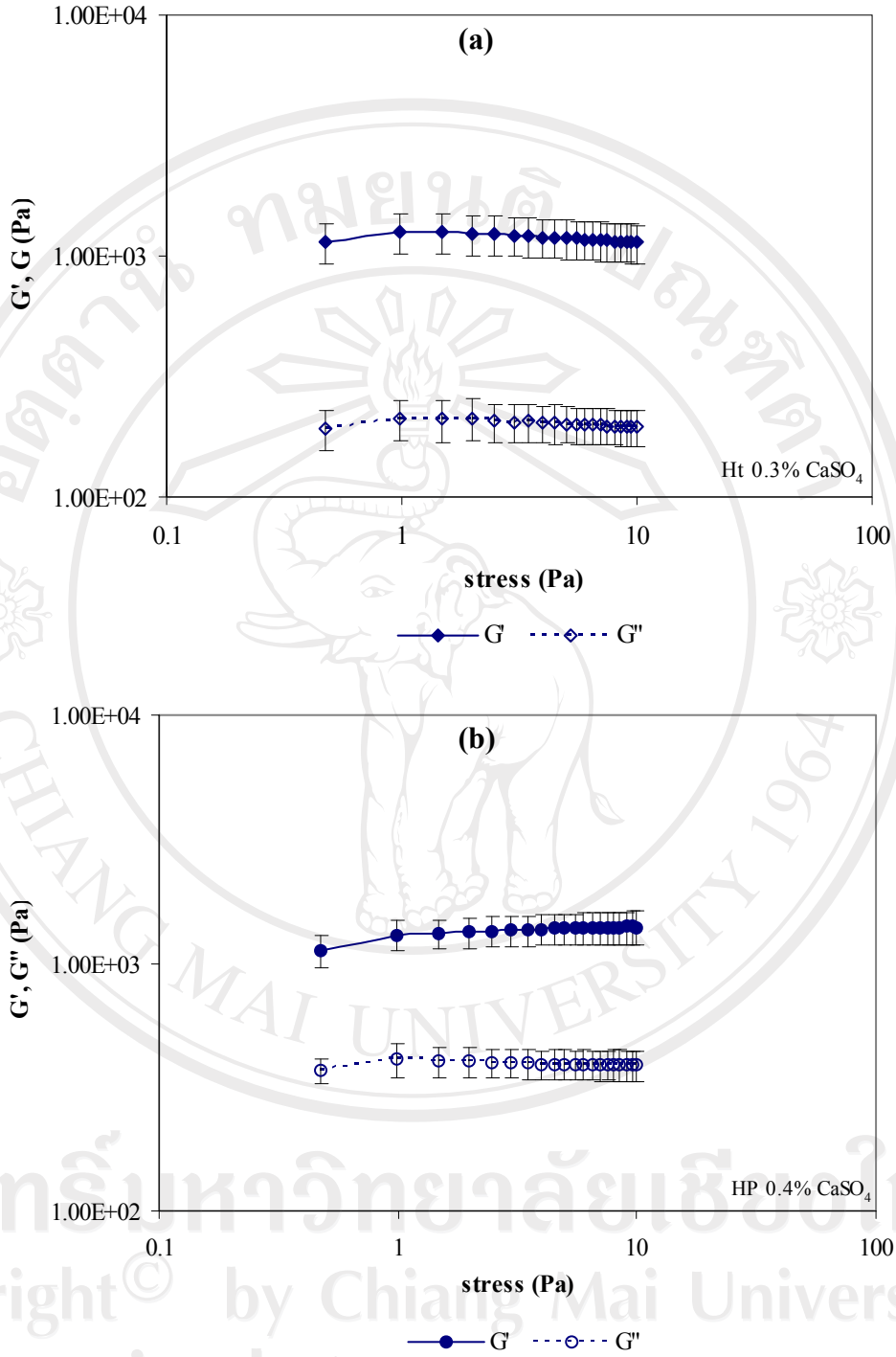

Figure 3.2 The storage (G') and loss (G'') moduli of $CaSO_4$ tofu gels as a function of the stress amplitude (0.5 to 10 Pa) at a frequency of 1 Hz (mean, n = 6) for: (a) 0.3% w/v $CaSO_4.2H_2O$ heat induced tofu gel; (b) 0.4% w/v $CaSO_4.2H_2O$ high pressure (400 MPa and 20°C for 10 min) induced tofu gel.

Figure 3.1 and 3.2 show the linear viscoelastic behaviour of both a 'soft' and a 'strong' sample prepared in this series of experiments. The plots of both the moduli of every sample examined showed the storage modulus predominating over the loss modulus over all of the measured amplitude range. These profiles also show the materials to behave in a linear viscoelastic manner well above the stress used for their subsequent rheological measurement. In such a linear region of behaviour the rheological properties are essentially strain or stress independent (Steffe, 1996).

Based on these results, a stress amplitude of 1 Pa was chosen as suitable to measure the oscillation frequency sweep of both Ht and HP induced tofu samples, this being well within the linear region of behaviour of all of the samples examined (Ferry, 1980).

The oscillation sweep measurement was measured over a frequency range of 0.01 to 10 Hz for each type of material under test. The reported storage (G') and loss (G") curve was constructed from the numerical average of six reproducible measurements (n = 6).

3.2.6 Confocal scanning laser microscopy (CSLM) of tofu

Sections of the samples (about 3 mm cube) were cut with a razor blade, placed on a microscope slide and a drop of fluorescent dye mixture of Fast green FCF and Nile Red solution dissolved in polyethyleneglycol 200 was added (Auty *et al*., 2001). CSLM work was performed using a Leica DM IRE 2 Confocal Scanning Microscope configured with an inverted microscope stage using a Helium/Neon laser filtering emission configuration (625-754 nm). Images of representative areas of each sample were taken using a magnification objective of 63×.

3.2.7 Water holding capacity of tofu

Water holding capacity (WHC) determinations were based on the method proposed by Molina *et al*. (2002) with some slight modification. Cylindrical portion (about 1 cm length and 9 mm diameter) of the tofu gel was placed into a centrifugal filter device (amicon Ultrafree®-Cl) pore dia 0.45 μm. A centrifugal force of 4000 g

was applied for 10 min at 15°C (Sorvall® RC 5 B plus, DuPont, USA) and the mass of released water measured. Three replicates of each gel were examined and the mean reported as the result from two different preparations (n = 6 in total). WHC was calculated as the percentage of water released per gram of initial sample.

3.2.8 Native polyacrylamide gel electrophoresis of tofu

The polyacrylamide gel electrophoresis was carried out using the method described by Laemmli (1970) without adding SDS or any type of reducing reagent, to investigate effects of high pressure on the noncovalent interactions present (i.e. electrostatic interactions, hydrophobic interactions and hydrogen bonds) and any existing disulfide bonds among the soy protein subunits. The separating gel was 7.5% acrylamide; with a stacking gel made using 4.5% acrylamide. A 10% gels has been reported to be suitable for proteins in the range of 15-200 kDa and a 5% gel has been suggested for proteins in the range of 15-350 kDa (Dunn, 1993). Therefore, 7.5% gels is likely to separate proteins in the range of about 15-270 kDa.

Both soymilk and tofu samples were freeze dried to concentrate protein in the samples prior to examination by electrophoresis. The proteins in the dried and finely ground samples were dissolved in 0.124 M Tris buffer (pH 7.5) for 3 hr and solicated for 5 min. They were then centrifuged 8510 g for 40 min. Supernatants were then mixed with a half volume of the sample buffer (0.124 M Tris buffer pH 6.8, containing 0.002% w/v bromophenol blue and 20% v/v glycerol). The buffer and both the running and stacking gels were prepared in the absence of SDS.

The sample 10 µl was applied to each well of the gel. Molecular weights were determined using "nondenatured" protein molecular weight markers (Sigma®, USA). Separation was carried out at a constant current of 25 mA and a maximum voltage of 250 V (using a vertical electrophoresis unit, Hoefer mini VE, Amercham Biosciences) until the dye reached to the bottom edge of the gel (about 1 hr 10 min). Fixing of the protein was done by immersing the gel in a 12% w/v trichloroacetic acid for 1 hr. The gels were subsequently stained using a solution of coomasie brilliant blue G-250 (80 ml of 0.1% w/v phosphoric acid, 10% w/v ammonium sulphate and

20 ml methanol adjusted to a final volume of 100 ml) (Neuhoff *et al*., 1988). After staining overnight, the gels were destained with a solution containing 20% v/v methanol in distilled water.

3.2.9 Statistical analysis

All composite data points represent the mean ± standard deviation. The means of two sample groups were compared by Paired T test. To analyse more than two treatments, an analysis of variance (ANOVA) using a General Linear Model was performed. When differences were observed, a Duncan's New Multiple Range Test (DMRT) with a confidence interval of 95% was used to compare the sample means. The analysis was carried out using software, SPSS 12.0 for Windows (SPSS Inc., Chicago, USA).

3.3 Results and discussion

3.3.1 Analysis of MF-soybeans, soymilks, and tofu

3.3.1.1 Composition of MF-soybeans and soymilks

Table 3.1 shows analytical results for MF-soybeans and their soymilk derivatives. Main components of the soybean and the soymilk are protein, carbohydrate, and fat. All analytical results were well within the range of values normally observed for soybean and soymilk samples, previously reported (Shen *et al*., 1991; Liu, 1999).

Table 3.1 The MF-soybeans and soymilks composition.

Determination	Soybean	Soymilk
Seed size (g/100 beans)	13.34 ± 0.37	-
Moisture content (%) [a]	9.80 ± 0.16	91.32 ± 0.34
Total solid (%) [a]	-	8.68 ± 0.34
pH value	-	6.4-6.6
Total protein (%) (N × 6.25)[a]	36.44 ± 0.49	4.43 ± 0.10
Total fat (%) [a]	11.14 ± 0.32	0.90 ± 0.03
Ash (%)[a]	4.65 ± 0.02	0.39 ± 0.004
Total carbohydrate (%)[a]	37.96	2.96
Water uptake (%)	240	-

[a] wet weight basis, n = 3

3.3.1.2 Activity of trypsin inhibitors in soybean and soymilks

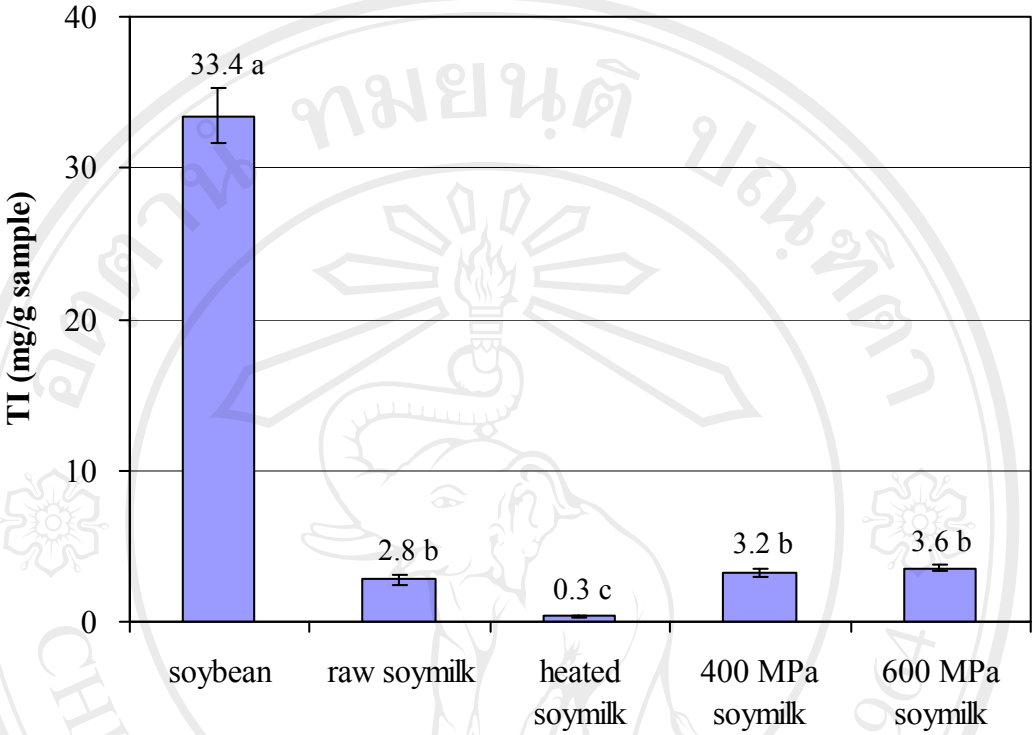

Figure 3.3 Activity of trypsin inhibitors in MF-soybean, raw soymilk, heated soymilk, and pressurised (400 MPa, 600 MPa) soymilks. Means followed by the same letters (a, b, c) are not significantly different ($p<0.05$).

Figure 3.3 shows that there was about 10% TI left in the raw soymilk after passing through different steps of extraction from soybean such as washing, soaking and diluting process. Prinyawiwatkul *et al*. (1996) found that soaking cowpea reduced TI activity by 20%, whereas boiling of soaked seeds decreased TI activity by 85%.

After heating at 97-100°C for 7 min, the TI activity of soymilk markedly reduced approximately to 1%. Whereas pressurisation at 400 and 600 MPa with 20°C for 10 min, the soymilk remained their TI activity at a similar level to that found for the untreated soymilk sample.

The extent of destruction of trypsin inhibitors in soymilk for maximum nutritive value or protein efficiency ration was reported to be 80-90% (Hackler *et al*., 1965) while a later work suggested that the time/temperature requirement be based on 85% deactivated of the trypsin inhibitors (Wilson, 1989; cited in Liu, 1999). Deactivation of trypsin inhibitors from soybeans and soymilks to reach 90% inactivation have been reported variously as, 100°C for 30 min or 110°C for 22 min (Liu, 1999) or ultrahigh-temperature (UHT) of 143°C for 62 sec (Kwok *et al*., 2002).

Although heat treatment inactivated TI in soymilk, extended heating should be avoided, because overheating leads to destruction of nutrients and essential amino acids and vitamins (Kwok *et al*, 2002). Extended heating also alters the functional properties of soy protein to such an extent that they become less coagulated when made into tofu (Liu, 1999). Beddow and Wong (1987) stated that heating soymilk at 100°C for 3 min was probably about the minimum needed to inactivate the trypsin inhibitors in soymilk.

The effectiveness of heat treatment on trypsin inhibitors also depends on the chemical form of proteins. The soy proteins, including trypsin inhibitors, become more resistant to heat treatment when in the form of heat extract. A possible explanation is that protein molecules are fully hydrated in the soymilk. They form many hydrogen bonds with water molecules. As a result, the tertiary and quaternary structure of soy protein becomes less easily disturbed by heat (Liu, 1999). DiPietro and Liener (1989) reported that when solutions of KTI, BBI, and crude extracted raw soy flour were heated (100°C), the soy extract lost inhibitory activity most rapidly, while purified KTI lost inhibitory activity at an intermediate rate and purified BBI lost inhibitory activity very slowly. When the inhibitors are in their pure form or in situ and were heated (75-95°C) within a soy flour matrix, soybean protease inhibitors still lost inhibitory activity most rapidly but purified BBI was inactivated more quickly than purified KTI.

Inactivation of trypsin inhibitor activity by high pressure combined with heat treatment was reported by van der Ven *et al*. (2005) at a high pressure of between 750 and 525 MPa at between 77 and 90°C 90% of trypsin inhibitor activity was lost in soymilk. They also reported that trypsin inhibitors might be rather stable to high

pressure at room temperature due to their conformation stability of the protein caused by the pressure of disulphide bonds (van der Ven *et al*., 2005).

3.3.1.3 Surface hydrophobicity of soymilk

Surface hydrophobicity of protein depended on amino acid composition and structural configuration, including the unfolding of the protein native structures (Zhang *et al*., 2003). Hydrophobic interactions play substantial role in stabilization of the tertiary structure and in protein interactions (Zhang *et al*, 2005). ANS is known to bind hydrophobic areas of proteins accessible to the aqueous solvent. Upon binding, its fluorescence is drastically enhanced so that exposed hydrophobic surface areas can be quantitatively determined (Gaucheron *et al*., 1997).

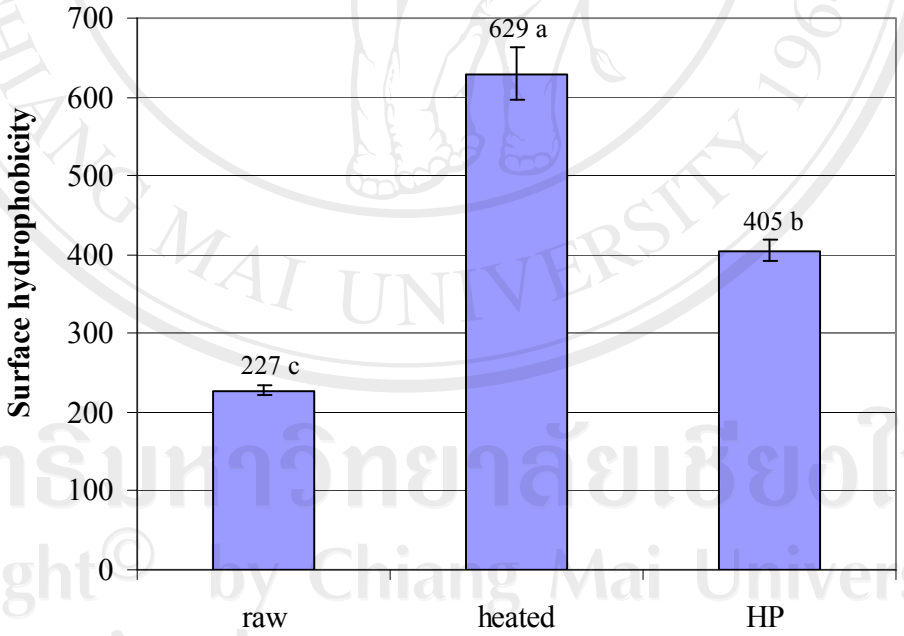

Figure 3.4 Surface hydrophobicity (So) of raw soymilk, heated soymilk, and pressurised (400 MPa 20°C 10 min) (HP) soymilk. Mean followed by the same letters (a, b, c) are not significantly different ($p<0.05$).

Figure 3.4 illustrates the hydrophobicity of raw, heat (Ht) and pressure (400 MPa 20°C 10 min) (HP) treated soymilks. The Ht soymilk exposed hydrophobic groups more than the HP and raw soymilk samples ($p<0.05$), respectively. The surface hydrophobicity of the Ht and HP soymilk were developed from raw soymilk, increasing 2.8 and 1.8 time, respectively, suggesting that heat and high pressure treatment induced a molecular unfolding of the protein with the exposure of the hydrophobic groups to the surface. The surface hydrophobicity was more pronounced in the heat treated sample, suggesting that under the experimental conditions used denaturation by heat is more severe than that of pressure. Zhang *et al*. (2005) also observed this phenomenon.

In raw soymilk, soy protein molecules maintain their native state with the hydrophobic regions located inside, which is in agreement with the typical globular structure of a native protein. Upon heating, the protein molecules unfold and their hydrophobic groups become exposed to the outside, resulting in increased surface hydrophobicity (Kohyama and Nishinari, 1993; Wu *et al*., 1998; Sorgentini *et al*., 1995) which exists more than 50°C heating temperature (Obata and Matsura, 1993). Studies on purified soy protein fraction indicated that heating at 70°C (Kato *et al*., 1983) and 85°C (Belyakova *et al*., 1999) caused a significant increase in surface hydrophobicity of both the 7S and 11S globulins, respectively.

Unlike heat, high pressure develops the surface hydrophobicity of molecules differently. The increase in surface hydrophobicity following high pressure treatment was observed for both the purified 11S and 7S globulins (Pedrosa and Ferreira, 1994; Zhang *et al*., 2003). Molina *et al*. (2001) observed an increase in surface hydrophobicity of pressurised soy protein isolate (SPI), 7S, and 11S globulins above 200 MPa at neutral pH which was a partial denaturation process. Whereas Puppo *et al*. (2004) observed a significant increase in surface hydrophobicity of pressurised materials (400 MPa) SPI at acidic pH (pH 3) and alkaline pH (pH 8).

3.3.1.4 Visual appearance and chemical analysis of gelled tofu systems

3.3.1.4.1 Heat induced tofu gels

Heat induced (Ht) tofu gels with added 0.2%-0.6% w/v GDL, 0.3%-0.6% w/v $CaSO_4.2H_2O$, and 0.13%-0.15% w/v $CaCl_2.2H_2O$ were smooth and homogenous in appearance (Figure A1 in appendix A).

The raw soymilk and preheated soymilk (97-100°C for 7 min) without coagulant, heated at the coagulation temperature used for Ht tofu production (70°C for 60 min), were still liquid. This suggested that the MF-soymilk (4.4% protein, 8.7% total solid) had too low a total protein concentration to form a gel without the use of a coagulant.

Protein concentration is a critical factor to form a 'self-standing' gel network, a minimum protein concentration; known as least concentration endpoint (LCE) is required. The LCE is at least 8% for soy protein (Damodaran, 1996). Hermansson (1978) reported that the minimum protein concentration for gelling is about 8% in distilled water at pH 7. Puppo and Anon (1998) stated that the minimum protein concentration required for heat induced gelation (90°C for 30 min) from the soy protein isolate (SPI) was around 7% w/w. Molina and Ledward (2003) reported that SPI and the 7S globulin at a concentration of 12% w/v protein, at neutral pH, did not form a gel when heat-treated at 90°C for 15 min, but the 11S globulin did.

3.3.1.4.2 High pressure induced tofu gels

The raw soymilk without coagulant remained liquid after processing at up to 700 MPa and at the various times and temperatures, shown in Table 3.2.

Table 3.2 Visual appearance of pressurised raw soymilks

Treatment	Visual appearance
400 MPa 20°C 10 min	liquid
500 MPa 20-40°C 10-40 min	liquid
600 MPa 20-40°C 10-40 min	liquid
700 MPa 20°C 10 min	liquid

However when the raw soymilk with added coagulant, (0.2% w/v GDL, 0.4% w/v GDL, 0.4% w/v calcium sulphate) was processed at 400 MPa and 20°C for 10 min gels were formed with a tendency to show quite, noticeable syneresis. The tofu samples with the same concentration of coagulant processed at higher pressures, longer times or higher temperatures became noncontinuous aggregates surrounded by an aqueous medium.

Molina *et al*. (2002) reported that high pressure alone could not form self-supporting gels for soy protein isolate (SPI), 7S, and 11S globulin at protein concentration lower than 20%. Gelation of tofu under high pressure treatment may be formed by soy protein denaturation caused primarily by high pressure; coagulation is also promoted by protons from coagulant. The hydrophobic region of the native protein molecules are exposed to the solvent by high pressure treatment. As the denatured soy protein is negatively charged (Kohyama and Nishinari, 1993), the protons produced by GDL or calcium ions neutralized the soy proteins which becomes less 'repulsive' and induced aggregation to form a cross-link network (Zhang *et al*., 2005). Some other interactions such as the oxidation of sulphydryl groups are also involved (Apichartsrangoon, 2003).

Okamoto *et al*. (1990) reported that a minimum pressure of 300 MPa with holding time for 10-30 min is needed to induce high pressure set soy protein gel. Zhang *et al*. (2005) reported that the soymilk was transformed from liquid to sol beyond pressure treated at 500 MPa for 30 min. The phase change of soymilk after high pressure treatments indicated that the soy proteins in soymilk had been modified

to form a colloidal phase. However the pH values of soymilk were not significances different after high pressure treatments.

Table 3.3 shows analytical results of the heat (Ht) induced and high pressure (400 MPa and 20°C for 10 min) (HP) induced tofu with added various coagulants.

Table 3.3 Chemical properties of heat induced (Ht) and high pressure induced (HP) tofu with added 0.4% w/v GDL, 0.4% w/v $CaSO_4.2H_2O$, and 0.14% w/v $CaCl_2.2H_2O$.

Tofu type	% Moisture content [a]	% Total protein [a]	pH range [b]
Ht-GDL	91.35 ± 0.19	4.63 ± 0.17	5.4-5.7
Ht-$CaSO_4$	91.42 ± 0.12	4.47 ± 0.11	5.9-6.2
Ht-$CaCl_2$	90.70 ± 0.61	4.45 ± 0.02	5.7-6.0
HP-GDL	86.64 ± 0.96	5.80 ± 0.04	5.2-5.4
HP-$CaSO_4$	86.75 ± 1.33	5.52 ± 0.00	5.6-6.0

[a] wet weight basis for means ± sd, n = 3

[b] n = 6

Total protein levels of tofu samples were similar to those of the protein values of the raw soymilk because the tofu samples were prepared by coagulating the soymilk without elimination of whey. Apart from protein, other components such as total fat and carbohydrate content of tofu samples were assumed to be similar to those in the original soymilks. The pH of the prepared tofu samples were found to be lower than that of the soymilk used. On addition of GDL, the pH of soymilk was lower because in solution GDL was hydrolysed into gluconic acid ($C_6H_{12}O_7$). While addition of calcium chloride and calcium sulphate into soymilk, calcium ions (Ca^{2+}) was liberated, these calcium ion would interact with phosphate ion in phytate and hydrogen ions was liberated, the calcium ion also interacted with carboxyl and imidazole groups of proteins. These interactions would gradually decrease the pH of

soymilk (Ono *et al.*, 1993). Saio (1979) also suggested that soybeans were found to contain about 600 mg of phosphorus, 70-80% of which was found to exist as phytic acid. Most of the phytic acid in seeds is extractable into the soymilk and co-precipitates with the other proteins in tofu during coagulation. Phytic acid reacts with both the calcium and the protein present and produces a colloidal precipitate that can hold elevated levels of moisture in the final gel (Saio, 1979).

3.3.2 Rheological properties of heat induced and high pressure induced tofu gels

3.3.2.1 Typical rheological behaviour of heat induced tofu gels

3.3.2.1.1 Heat induced GDL tofu gels

Figure 3.5 shows the mean storage (G') and loss (G") moduli of a heat induced tofu with 0.2% to 0.6% w/v added GDL, as a function of the frequency (0.1 to 10 Hz), at a stress amplitude of 1 Pa.

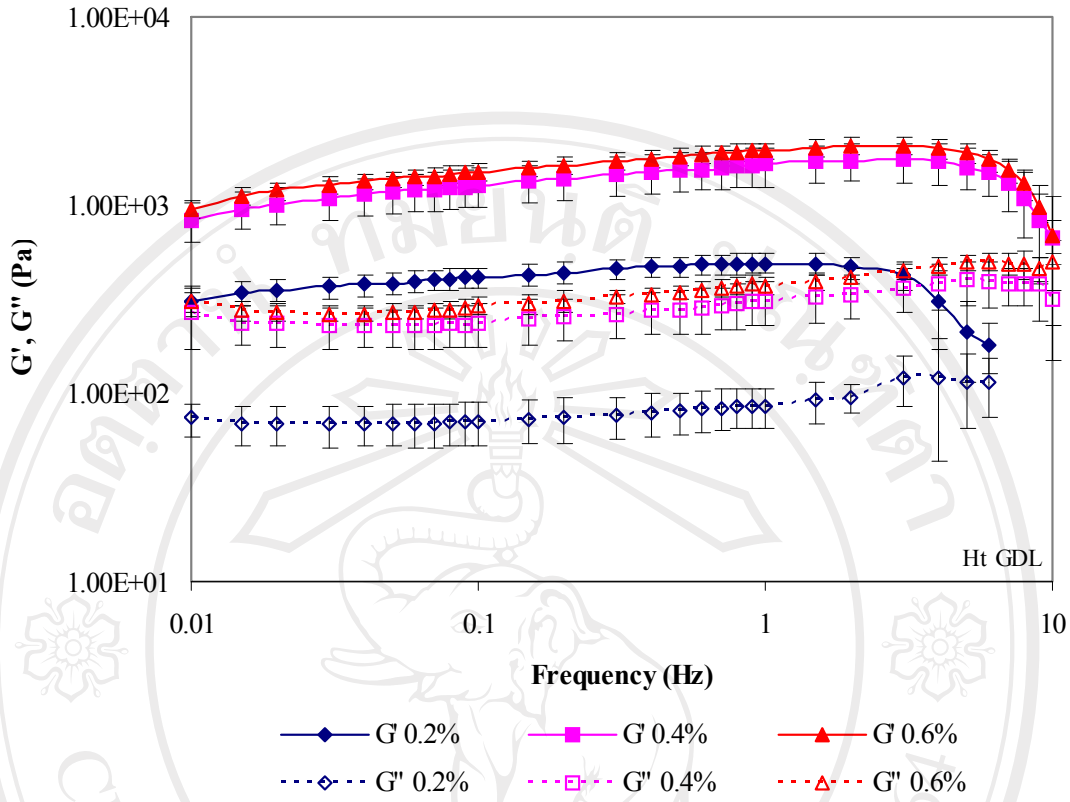

Figure 3.5 The storage (G') and loss (G'') moduli of heat induced tofu with 0.2% to 0.6% w/v added GDL as a function of the frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

The storage moduli of heat induced 0.2% to 0.6% w/v GDL tofu gels showed the elastic or storage moduli (G') values to be greater than viscous or loss moduli (G'') values measured over all of the assessed frequency range. Such profiles are consistent with the behaviour of a material with weak non-specific interactions giving rise to amorphous gel structure (Ferry, 1980).

All of the samples showed a slight increase with increasing frequency and had some evidence of structural break down at some of the higher frequencies. Lower concentrations of the coagulants showed this structural break down at progressively lower frequencies than those of the higher coagulant concentrations. These results implied that at lower GDL concentrations, the interaction of the protein molecules

with protons from the coagulant was not as strong as those formed at higher GDL concentrations.

The frequency sweep for Ht GDL tofu gels showed significance increases in the rheological properties (moduli values) with increasing GDL from 0.2% to 0.4% w/v indicated that at the 0.4% w/v GDL is stronger. The relatively small changes observed between the samples made with soymilk added GDL level of 0.4-0.6% suggested that a 'saturation point' or 'plateau' was being reached. The higher concentration of GDL (0.8% w/v) used in these section gave rise to distinct sample curding during mixing and created 'lumpy' product on coagulation step.

Based on these result the concentration using a concentration of 0.4% w/v GDL was selected for the all further experimental gels.

Table 3.4 Viscoelastic values observed at 0.1 and 1.0 Hz of heat induced tofu gels with 0.2% to 0.6% w/v GDL added.

Tofu type	**G' (kPa)**	**G" (kPa)**	**tan δ**
0.1 Hz			
0.2% GDL	0.41 ± 0.05 a	0.071 ± 0.019 a	0.17 ± 0.03
0.4% GDL	1.27 ± 0.30 b	0.237 ± 0.060 b	0.19 ± 0.01
0.6% GDL	1.50 ± 0.15 b	0.289 ± 0.031 c	0.19 ± 0.01
1.0 Hz			
0.2% GDL	0.49 ± 0.60 a	0.086 ± 0.021 a	0.17 ± 0.02
0.4% GDL	1.64 ± 0.39 b	0.307 ± 0.080 b	0.19 ± 0.01
0.6% GDL	1.95 ± 0.19 b	0.374 ± 0.039 c	0.19 ± 0.01

The results are the means ± standard deviations of six replicates. Different letters (a, b, c) indicate significant ($p<0.05$) differences within a column of individual frequency (0.1, 1.0 Hz).

Table 3.4 illustrate the loss tangent (tan δ, G”/G’) of heat induced GDL tofu gels lay in the range 0.17-0.19 at 0.1 and 1.0 Hz, this behaviour is a typical of a ‘weak’ viscoelastic gel type material, with the loss tangent approaching unity (Ferry, 1980)

3.3.2.1.2 Heat induced tofu $CaSO_4$ gels

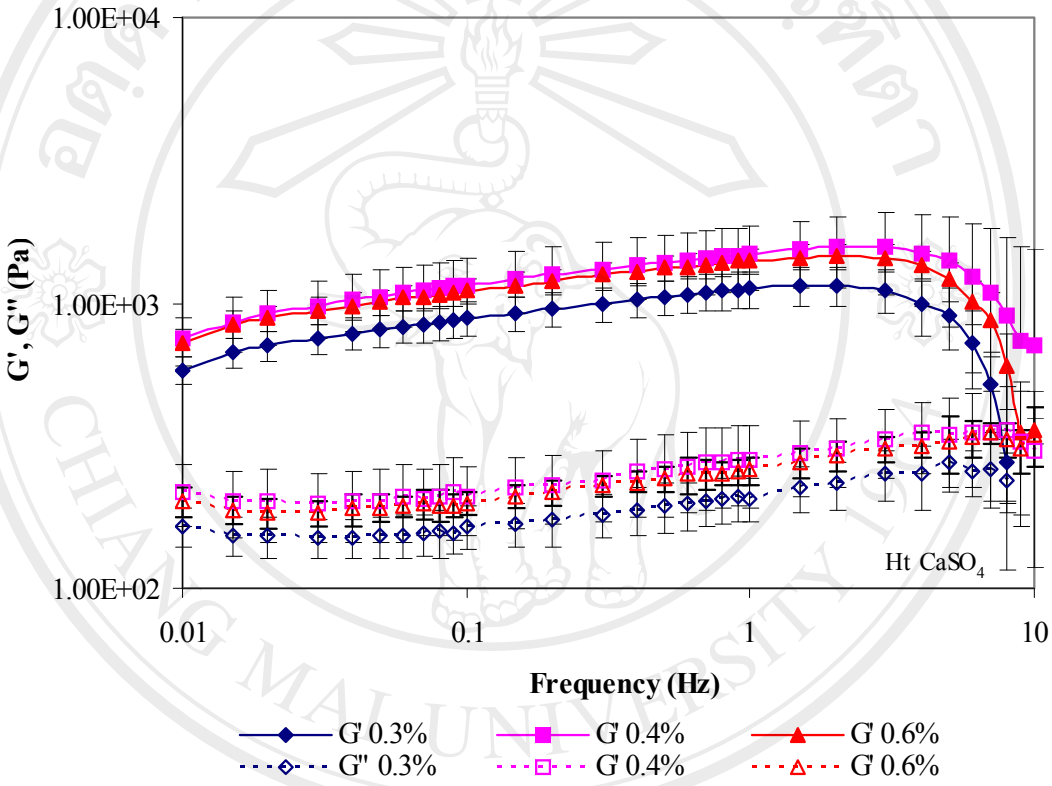

Figure 3.6 The storage (G’) and loss (G’’) moduli of heat induced tofu gels with 0.3% to 0.6% w/v added $CaSO_4.2H_2O$ as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

Figure 3.6 shows the moduli of tofu gels with different concentrations of $CaSO_4.2H_2O$ gave essentially the same overall rheological profiles. All samples gave frequency profiles expected for weak viscoelastic materials (elastic or storage modulus (G’) greater than the viscous or loss modulus (G”) over all of the measured frequency range) (Ferry, 1980).

The moduli of the Ht $CaSO_4.2H_2O$ tofu showed an increase in the rheological properties with increasing coagulant concentration from 0.3% to 0.4% w/v, but the moduli of 0.4% and 0.6% w/v $CaSO_4.2H_2O$ samples were similar in value. However the samples although having different overall consistencies the samples with different concentrations of $CaSO_4.2H_2O$ all gave similar loss tangent values (0.18 to 0.19) as shown in Table 3.5. This result suggested that the $CaSO_4.2H_2O$ tofu samples displayed essentially a similar amorphous gel type structure (Ferry, 1980).

Similar to the Ht tofu gels with added GDL (Figure 3.5), the Ht tofu gels with added $CaSO_4.2H_2O$ showed had some evidence of structural break down at some of the higher frequencies. Lower concentrations of the coagulants showed this structural break down at progressively lower frequencies than those of the higher coagulant concentrations. These results implied that at lower $CaSO_4.2H_2O$ concentrations, the interaction of the protein molecules with protons from the coagulant was not as strong as those formed at higher $CaSO_4.2H_2O$ concentrations.

The higher concentration of $CaSO_4.2H_2O$ (0.8% w/v) used in these section gave rise to some sample curding during mixing. This eventually created very different 'lumpy' final products. Based on these results the concentration at 0.4% w/v $CaSO_4.2H_2O$ was selected for the all further experimental gels.

Table 3.5 Viscoelastic values observed at 0.1 and 1.0 Hz of heat induced tofu gels with 0.3% to 0.6% w/v levels of added $CaSO_4.2H_2O$.

Tofu type	G' (kPa)	G" (kPa)	tan δ
0.1 Hz			
0.3% $CaSO_4.2H_2O$	0.88 ± 0.12 ns	0.17 ± 0.03 ns	0.19 ± 0.01
0.4% $CaSO_4.2H_2O$	1.16 ± 0.27 ns	0.21 ± 0.56 ns	0.18 ± 0.01
0.6% $CaSO_4.2H_2O$	1.10 ± 0.11 ns	0.20 ± 0.19 ns	0.18 ± 0.01
1.0 Hz			
0.3% $CaSO_4.2H_2O$	1.13 ± 0.16 a	0.21 ± 0.37 ns	0.18 ± 0.01
0.4% $CaSO_4.2H_2O$	1.49 ± 0.38 b	0.28 ± 0.09 ns	0.19 ± 0.01
0.6% $CaSO_4.2H_2O$	1.41 ± 0.14 ab	0.26 ± 0.03 ns	0.18 ± 0.01

The results are the means ± standard deviations of six replicates. Different letters (a, b) indicate significant ($p<0.05$) differences, ns indicates not significant ($p>0.05$) difference within a column of individual frequency (0.1, 1.0 Hz).

3.3.2.1.3 Heat induced $CaCl_2$ tofu gels

Figure 3.7 show the storage (G') and loss (G") moduli of heat (Ht) induced tofu gels containing 0.13% to 0.15% w/v $CaCl_2.2H_2O$, as a function of frequency.

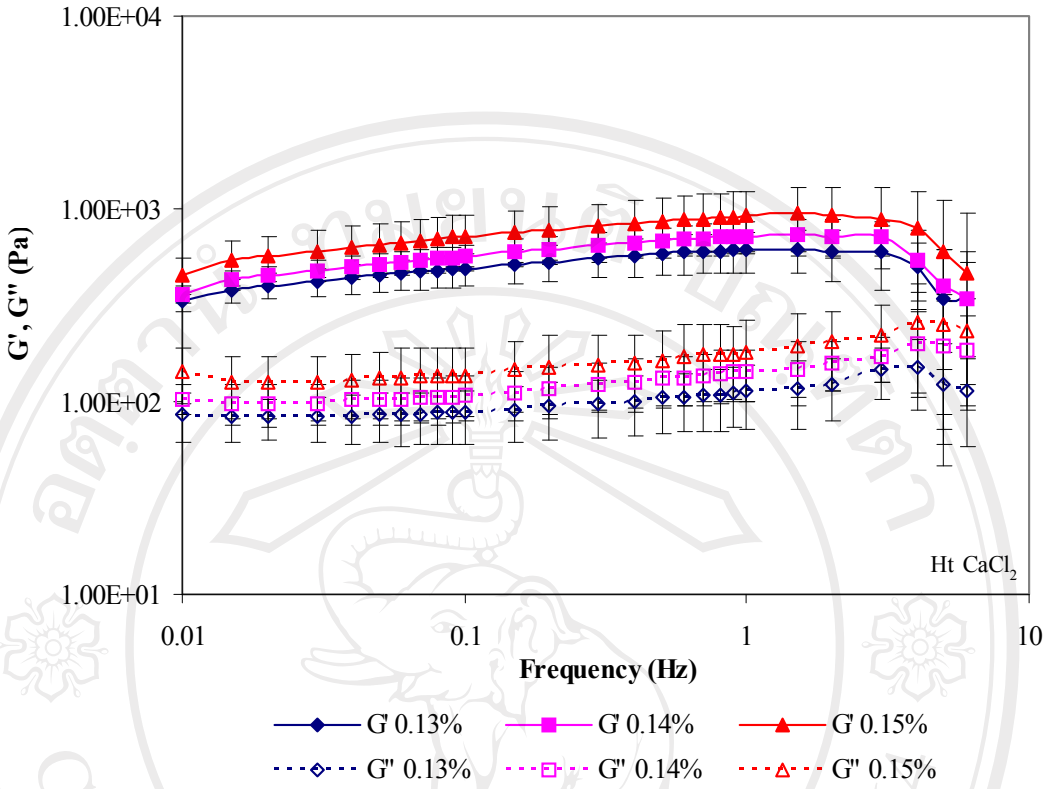

Figure 3.7 The storage (G') and loss (G'') moduli of heat induced tofu gels with 0.13% to 0.15% w/v added $CaCl_2.2H_2O$ as a function of frequency (0.01-6 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

The overall 'shape' of the Ht $CaCl_2.2H_2O$ tofu moduli profiles are similar to those obtained for the Ht GDL tofu (Figure 3.5) and the Ht $CaSO_4.2H_2O$ tofu preparations (Figure 3.6). All samples gave the frequency profiles expected for a 'weak' viscoelastic material (elastic or storage modulus (G') greater than the viscous or loss modulus (G'') over all of the measured frequency range). The storage moduli of the Ht tofu gel with added 0.15% $CaCl_2.2H_2O$ was higher than those with added 0.13% and 0.14% w/v $CaCl_2.2H_2O$. All of the Ht $CaCl_2.2H_2O$ tofu gels showed some structural break down at higher frequencies, the lower concentration of coagulant the 'earlier' the structural break down (lower frequencies than of the higher coagulant concentrations).

The loss tangent values (tan δ, G"/G') of the Ht $CaCl_2.2H_2O$ tofu were in the range 0.18-0.19 (Table 3.6) which again indicated 'amorphous' gel structure (Ferry, 1980). The Ht tofu materials made with higher concentrations of $CaCl_2.2H_2O$ show higher overall values of the storage (G') moduli than those made with the lower concentration of $CaCl_2.2H_2O$ indicating increasing gel 'strength' with increasing coagulant concentrations. Similarly, Maltais *et al*. (2005) reported that elastic modulus (G') of soy protein isolate gel with added $CaCl_2$ increased with increasing protein and calcium chloride concentration.

Base on these results a concentration of 0.14% w/v $CaCl_2.2H_2O$ was selected for use in the further experimental gels.

The concentration of $CaCl_2.2H_2O$ in preparation of tofu gels is limited to a small amount, since it is freely soluble in water and completely dissociated while $CaSO_4.2H_2O$ is partly dissociated in water (Windholz *et al*, 1983; Liu, 1999), therefore in setting tofu gel an excess amount is needed to induce gelation.

Table 3.6 Viscoelastic values observed at 0.1 and 1.0 Hz of heat induced tofu gels with 0.13% to 0.15% w/v added $CaCl_2.2H_2O$.

Tofu type	**G' (kPa)**	**G" (kPa)**	**tan δ**
0.1 Hz			
0.13% $CaCl_2.2H_2O$	0.49 ± 0.09 a	0.09 ± 0.03 a	0.18 ± 0.02
0.14% $CaCl_2.2H_2O$	0.57 ± 0.09 ab	0.11 ± 0.03 ab	0.19 ± 0.02
0.15% $CaCl_2.2H_2O$	0.71 ± 0.21 b	0.14 ± 0.05 b	0.19 ± 0.02
1.0 Hz			
0.13% $CaCl_2.2H_2O$	0.61 ± 0.15 a	0.11 ± 0.04 a	0.18 ± 0.02
0.14% $CaCl_2.2H_2O$	0.72 ± 0.14 ab	0.14 ± 0.04 ab	0.19 ± 0.02
0.15% $CaCl_2.2H_2O$	0.92 ± 0.31 b	0.18 ± 0.08 b	0.19 ± 0.02

The results are the means ± standard deviations of six replicates. Different letters (a, b) indicate significant ($p<0.05$) differences within a column of individual frequency (0.1, 1.0 Hz).

3.3.2.1.4 Heat induced tofu gels with mixed coagulant systems

Figure 3.6 shows the values of the storage (G') and loss (G") moduli of heat (Ht) induced tofu gels made with mixed coagulants, 0.2% w/v GDL + 0.2% w/v $CaSO_4.2H_2O$; 0.2% w/v GDL + 0.07% w/v $CaCl_2.2H_2O$; 0.2% w/v $CaSO_4.2H_2O$ + 0.07% w/v $CaCl_2.2H_2O$.

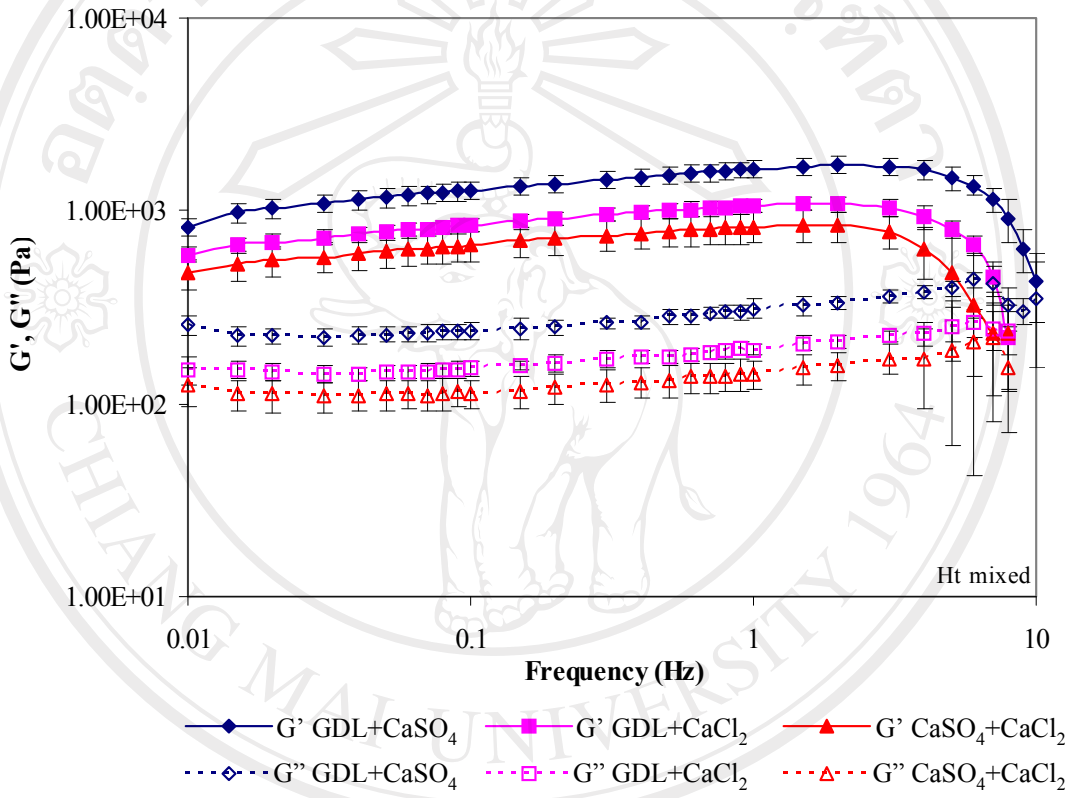

Figure 3.8 The storage (G') and loss (G") moduli of heat induced tofu gels with mixed coagulant as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

Moduli values of the gels made with mixed coagulants lie between the moduli values of those made with each of the individual component coagulants. The gels made with added 0.2% w/v GDL + 0.2% w/v $CaSO_4.2H_2O$ had significant highest G' and G" values when compared to those made with added 0.2% w/v GDL + 0.07% w/v $CaCl_2.2H_2O$ or 0.2% w/v $CaSO_4.2H_2O$ + 0.07% w/v $CaCl_2.2H_2O$. These

results corresponded well with the G' and G" value of tofu gels added each of single coagulant. These results suggest that there is no apparent synergy or interference of one coagulant with the other.

The overall values of the loss tangents at 0.1 and 1.0 Hz were in the range 0.17-0.19 (Table 3.7) which were similar to those of tofu samples coagulated with a single coagulant. These results suggest that the tofu prepared with the mixed coagulants displays essentially the same structure as those prepared with each of the single coagulants. This again suggests an 'amorphous' non-specific type of interaction for the gelling mechanism (Ferry, 1980).

Table 3.7 Viscoelastic values observed at 0.1 and 1.0 Hz for the heat induced tofu gels prepared with mixed coagulant.

Tofu type	G' (kPa)	G" (kPa)	tan δ
0.1 Hz			
GDL + $CaSO_4.2H_2O$	1.28 ± 0.14 c	0.24 ± 0.02 c	0.186 ± 0.008 b
GDL + $CaCl_2.2H_2O$	0.84 ± 0.07 b	0.15 ± 0.13 b	0.182 ± 0.003 b
$CaSO_4.2H_2O$ + $CaCl_2.2H_2O$	0.66 ± 0.12 a	0.11 ± 0.02 a	0.172 ± 0.008 a
1.0 Hz			
GDL + $CaSO_4.2H_2O$	1.65 ± 0.17 c	0.31 ± 0.04 c	0.188 ± 0.010 b
GDL + $CaCl_2.2H_2O$	1.06 ± 0.09 b	0.19 ± 0.01 b	0.180 ± 0.008 ab
$CaSO_4.2H_2O$ + $CaCl_2.2H_2O$	0.83 ± 0.15 a	0.14 ± 0.02 a	0.173 ± 0.007 a

The results are the means ± standard deviations of six replicates. Different letters (a, b, c) indicate significant ($p<0.05$) differences within a column of individual frequency (0.1, 1.0 Hz).

Ono *et al*. (1993) stated that the interaction of proteins in tofu made by calcium addition are considered to be firmer than that those formed by GDL addition. As the pH decrease in GDL system it aggregates the proteins by decreasing electric repulsion and liberates the 'hydration' or 'bound' water of the protein. While the

binding of calcium ions to carboxyl groups of the protein brings about association (Ono, *et al*., 1976 cited in Ono *et al*., 1993). Kohyama *et al*. (1995), inferred from their respective rheological properties, that the structure of calcium induced gels of tofu were quite similar to those of the GDL treated gels. The differences between the GDL and calcium sulphate induced gels were mainly due to rate of reaction of calcium sulphate (30 mM) faster than those GDL (20 mM) did (Kohyama *et al*., 1995). De Man *et al*. (1986) reported that $CaCl_2.2H_2O$ coagulated the soymilk almost instantly while GDL and $CaSO_4.½H_2O$ acted more slowly.

3.3.2.2 Typical rheological behaviour of high pressure induced tofu gels

3.3.2.2.1 High pressure induced GDL tofu gels

Figure 3.9 shows the storage (G') and loss (G") moduli of high pressure (HP) induced 0.2% w/v GDL tofu formed at 20, 30, and 40°C as a function of frequency (0.1 to 10 Hz), at a stress amplitude of 1 Pa.

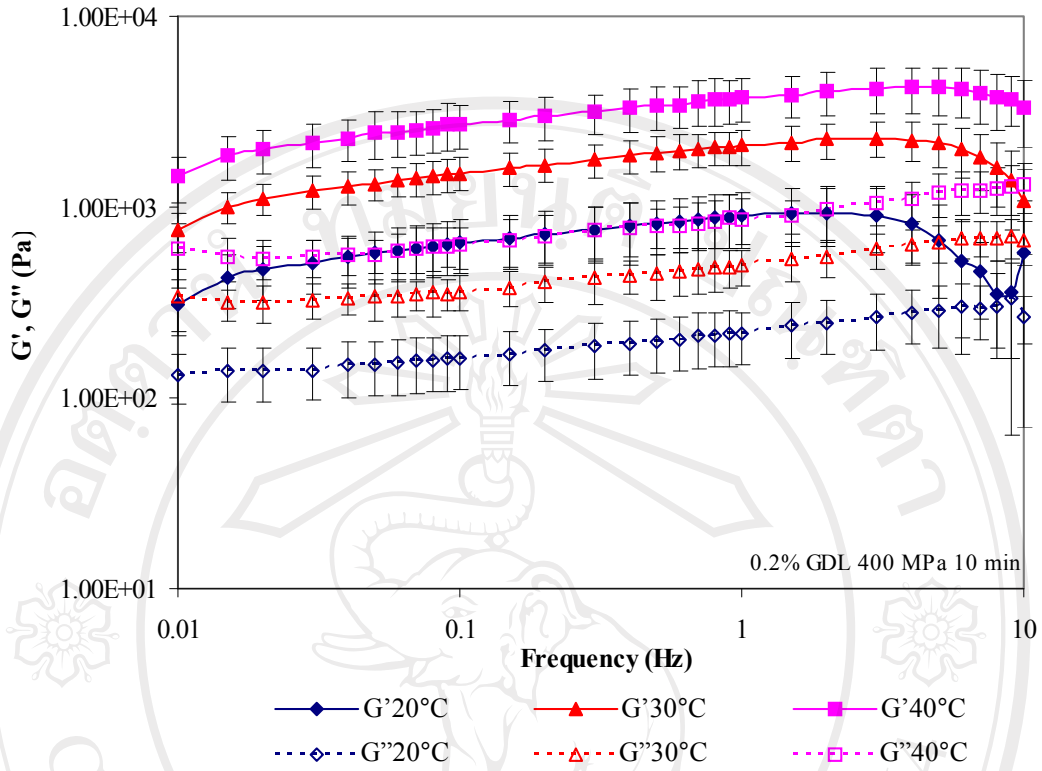

Figure 3.9 The storage (G') and loss (G") moduli of high pressure (400 MPa 10 min at 20, 30, and 40°C) induced tofu gels with 0.2% w/v GDL added as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

The storage (G') and loss (G'') moduli of the HP 0.2% w/v GDL tofu gels processed at 20, 30, and 40°C gave essentially the same rheological profiles (Figure 3.7). All gels gave frequency profiles expected for weak viscoelastic materials (elastic or storage modulus (G') greater than the viscous or loss modulus (G'') over all of the measured frequency range), the storage moduli slightly increasing with increasing frequency. The tofu gels processed at 30 and 40°C show higher storage (G') moduli than that those processed at 20°C but the samples processed at the higher temperatures showed increased syneresis and a 'loose' gel structure which was not expected for this type of tofu product.

Loss tangent (tan δ) values at 0.1 and 1.0 Hz lay in the range of 0.24-0.25 as shown in Table 3.8. Such profiles are consistent with the behaviour of a material with weak non-specific interactions giving rise to amorphous gel structures (Ferry, 1980).

Table 3.8 Viscoelastic values observed at 0.1 and 1.0 Hz for high pressure (400 MPa) induced tofu gels with 0.2% w/v GDL added, at various time and temperature conditions.

Tofu type	**G' (kPa)**	**G" (kPa)**	**tan δ**
0.1 Hz			
20°C 10 min	0.65 ± 0.20	0.16 ± 0.05	0.25 ± 0.004
30°C 10 min	1.51 ± 0.30	0.36 ± 0.08	0.24 ± 0.004
40°C 10 min	2.69 ± 0.69	0.63 ± 0.16	0.24 ± 0.02
20°C 20 min	0.34 ± 0.08	0.08 ± 0.02	0.25 ± 0.004
20°C 30 min	0.40 ± 0.13	0.10 ± 0.03	0.24 ± 0.003
1.0 Hz			
20°C 10 min	0.90 ± 0.29	0.22 ± 0.07	0.24 ± 0.01
30°C 10 min	2.10 ± 0.44	0.49 ± 0.11	0.23 ± 0.01
40°C 10 min	3.77 ± 1.01	0.87 ± 0.24	0.23 ± 0.02
20°C 20 min	0.46 ± 0.11	0.11 ± 0.02	0.24 ± 0.01
20°C 30 min	0.54 ± 0.19	0.14 ± 0.04	0.25 ± 0.01

The results are the means ± standard deviations of six replicates.

Increasing the processing time at high pressure (400 MPa 20°C) 0.2% w/v GDL for the tofu gels from 10 to 30 min, produced materials with essentially similar rheological profiles (Figure 3.10) with almost the same range of loss tangent values of

0.24-0.25 at 0.1 and 1.0 Hz (Table 3.8). The samples processed for 20 min and 30 min show structural break down at lower frequencies than those processed for 10 min.

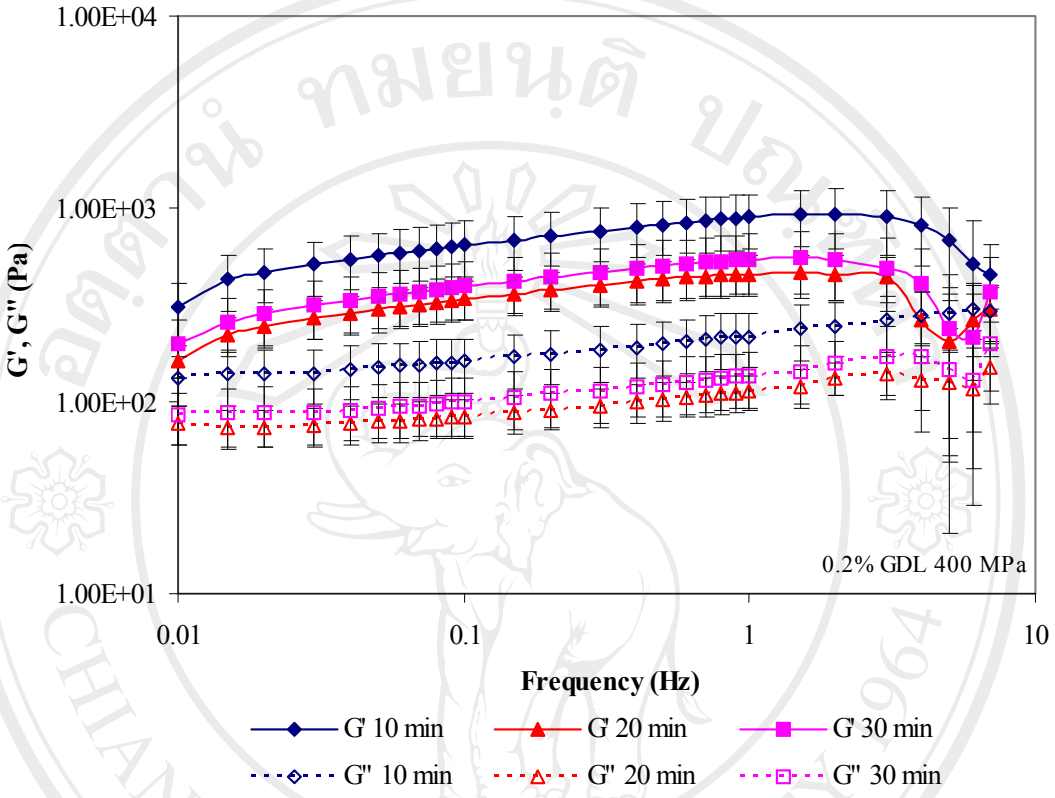

Figure 3.10 The storage (G') and loss (G") moduli of high pressure (400 MPa 20°C for 10 to 30 min) induced tofu gels with 0.2% w/v GDL added as a function of frequency (0.01-7 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

Figure 3.11 shows the storage (G') and loss (G") moduli of high pressure induced (HP) 0.4% w/v GDL tofu gels at 20, 30, and 40°C as a function of frequency (0.1 to 10 Hz, at a stress amplitude of 1 Pa).

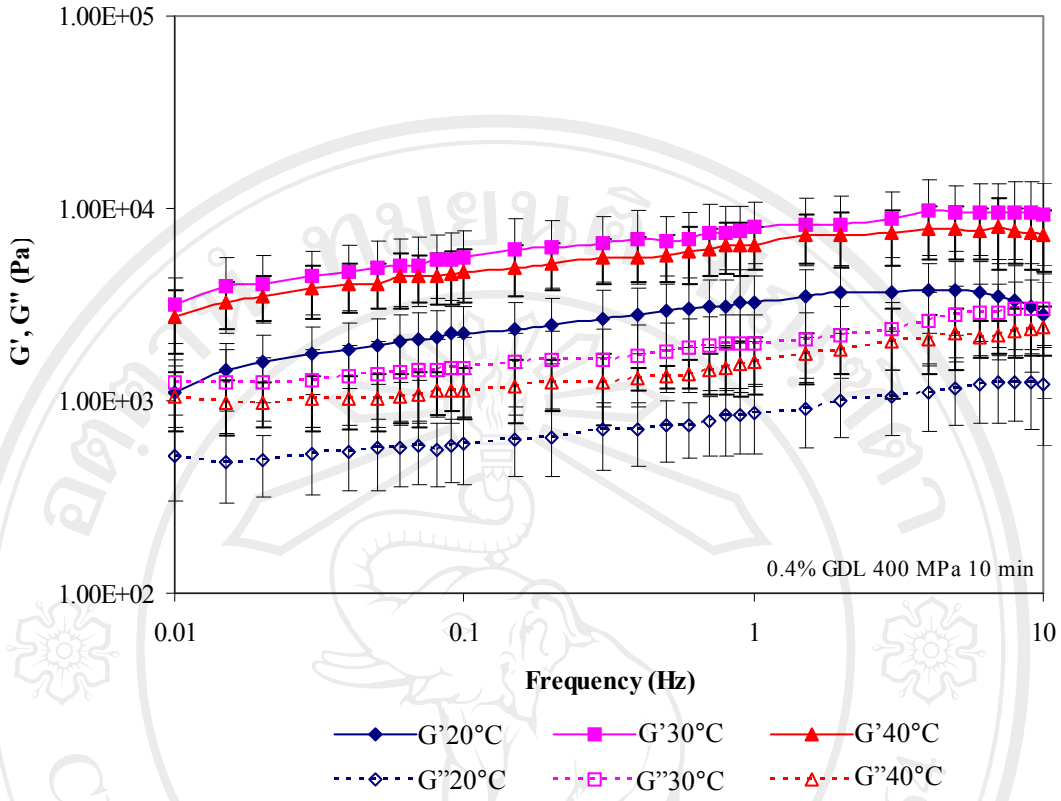

Figure 3.11 The storage (G') and loss (G") moduli of high pressure (400 MPa 10 min) induced tofu gels with 0.4% w/v GDL added at 20, 30, and 40°C as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

The HP (400 MPa 10 min) 0.4% w/v GDL tofu gels processed at 20, 30 and 40°C give essentially the same unchanging rheological profile with similar loss tangent values (0.24-0.27 at 0.1 and 1.0 Hz) as shown in Table 3.9. These are also similar to those of the HP 0.2% w/v GDL tofu prepared at the same temperature (Figure 3.9). However the HP 0.4% w/v GDL tofu materials showed higher moduli implying that the 0.4% w/v GDL produced an overall 'stronger' gel.

Table 3.9 Viscoelastic values observed at 0.1 and 1.0 Hz of high pressure (400 MPa) induced tofu gels at 20-40°C for 10 min with 0.4% w/v added GDL.

Tofu type	G' (kPa)	G" (kPa)	tan δ
0.1 Hz			
20°C	2.22 ± 0.83 a	0.60 ± 0.23 a	0.27 ± 0.02
30°C	5.54 ± 2.10 b	1.49 ± 0.66 b	0.26 ± 0.02
40°C	4.69 ± 1.45 b	1.13 ± 0.34 b	0.24 ± 0.01
1.0 Hz			
20°C	3.23 ± 1.19 a	0.86 ± 0.33 a	0.27 ± 0.01
30°C	8.00 ± 2.88 b	1.99 ± 0.79 b	0.25 ± 0.01
40°C	6.42 ± 1.68 b	1.61 ± 0.53 b	0.25 ± 0.02

The results are the means ± standard deviations of six replicates. Different letters (a, b) indicate significant ($p<0.05$) differences within a column of individual frequency (0.1, 1.0 Hz).

The experiment on high pressure induced tofu gel with added GDL in this section suggest that the tofu gels could be formed with whether 0.2% w/v GDL or 0.4% w/v GDL under pressure of 400 MPa for 10 min at room temperature. The HP 0.4% w/v GDL gels behave a stronger gel than the 0.2% w/v GDL gels. The treatments obtained from longer time and higher temperature resulted in a high level of syneresis and loose gels that were not expected for tofu type product.

3.3.2.2.2 High pressure induced $CaSO_4$ tofu gels

Figure 3.10 shows the storage (G') and loss (G") moduli of high pressure (HP) induced 0.4% w/v $CaSO_4.2H_2O$ tofu gels at 20 and 40°C as a function of frequency (0.1 to 10 Hz, at a stress amplitude of 1 Pa).

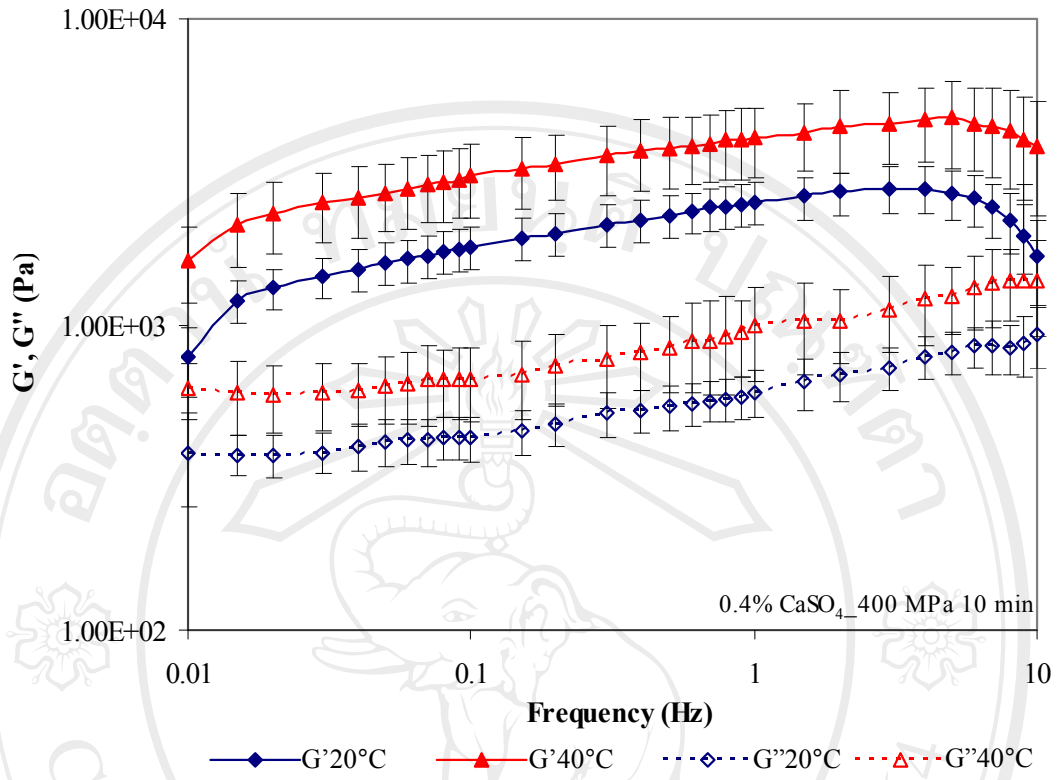

Figure 3.12 The storage (G') and loss (G") moduli of high pressure (400 MPa 10 min) induced tofu gels with 0.4% w/v added $CaSO_4.2H_2O$ at 20 and 40°C as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

The moduli of HP (400 MPa 10 min.) 0.4% w/v $CaSO_4.2H_2O$ tofu materials were similar to those of the HP GDL tofu samples with a similar range of loss tangent values (0.22 to 0.24 at 0.1 and 1.0 Hz) (Table 3.10). This result suggested the HP $CaSO_4$ tofu materials all display similar properties rheologically and that all of the samples were weak viscoelastic systems, with similar overall profiles. All of the HP 0.4% w/v $CaSO_4.2H_2O$ tofu samples showed some structural break down at the higher frequencies. The samples processed at 40°C showed higher moduli values, indicated stronger gels than those processed at 20°C. However the 40°C gel showed greater levels of synerisis and a 'crumbly' structure that were not expected for this type of tofu product.

Table 3.10 Viscoelastic values observed at 0.1 and 1.0 Hz of high pressure (400 MPa) set tofu gels at 20 and 40°C for 10 min with 0.4% w/v added $CaSO_4.2H_2O$.

Tofu type	G' (kPa)	G" (kPa)	tan δ
0.1 Hz			
20°C	1.77 ± 0.28	0.43 ± 0.07	0.24 ± 0.02
40°C	3.06 ± 0.82	0.66 ± 0.18	0.22 ± 0.02
1.0 Hz			
20°C	2.52 ± 0.41	0.60 ± 0.11	0.24 ± 0.01
40°C	4.07 ± 1.03	0.99 ± 0.34	0.24 ± 0.02

The results are the means ± standard deviations of six replicates.

3.3.2.3 Effects of temperature and high pressure on rheological properties of tofu gels

Figures 3.13 and 3.14 show the storage (G') and loss (G'') moduli of heat processed and high pressure processed tofu samples with 0.4% w/v added GDL and 0.4% w/v added $CaSO_4.2H_2O$ as functions of frequency.

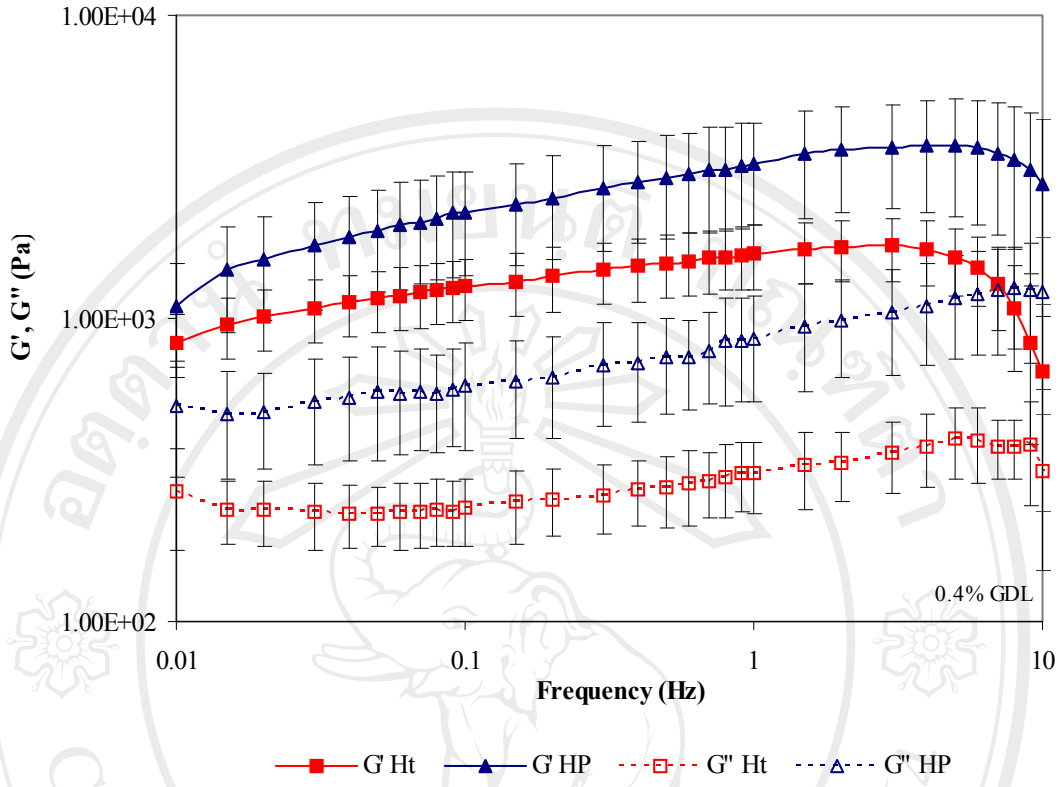

Figure 3.13 The storage (G') and loss (G'') moduli of heat (Ht) and high pressure (400 MPa 10 min) (HP) induced tofu gels with 0.4% w/v added GDL as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

The storage moduli of both the heat and high pressure induced GDL and $CaSO_4.2H_2O$ treated tofu gels increased slightly with increasing frequency. All samples gave the frequency profiles expected for weak viscoelastic systems for which the elastic or storage modulus (G') was greater than the viscous or loss modulus (G'') over most of the measured frequency range. Such profiles are consistent with the behaviour of a material with weak non-specific interactions giving rise to amorphous gel structures (Ferry, 1980).

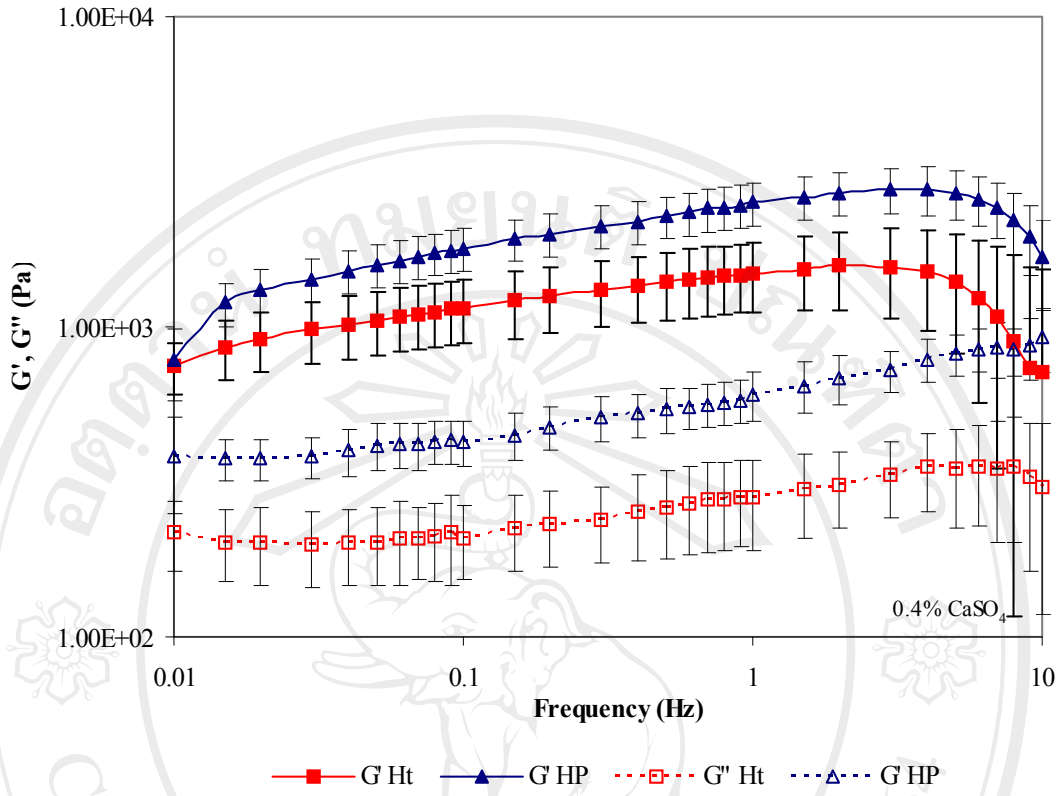

Figure 3.14 The storage (G') and loss (G") moduli of heat (Ht) and high pressure (400 MPa 10 min) (HP) induced tofu gels with 0.4% w/v added $CaSO_4.2H_2O$ as a function of frequency (0.01-10 Hz) at a stress amplitude of 1 Pa (mean, n = 6).

Table 3.11 Comparison of heat (Ht) and high pressure (400 MPa 20°C 10 min) (HP) induced tofu gels with 0.4% w/v added GDL and 0.4% w/v added $CaSO_4.2H_2O$ of mean values (n = 6) across frequency range of 0.01-6.0 Hz.

Coagulant	Paired means					
	G' (kPa)		G" (kPa)		Loss tangent	
	Ht	htHP	Ht	htHP	Ht	htHP
GDL	1.39	2.63 s	0.28	0.74 s	0.20	0.29 s
$CaSO_4.2H_2O$	1.25	2.05 s	0.25	0.53 s	0.21	0.26 s

s indicates a significant ($p<0.05$) difference between the paired means.

The frequency dependence profiles of the storage and loss moduli for all the differing types of tofu produced by the various methods were essentially the same. These similar rheological profiles suggested that although the absolute 'strength' of the tofu samples may vary, they were essentially the same type of rheological system, presumably formed by the non-specific cross linking of the denatured protein material. Kohyama *et al*. (1995) supported that the Ht GDL and calcium gels displayed similar breaking strain.

HP processed tofu produced with both GDL and calcium sulphate give significantly higher values of both the storage (G') and the loss (G'') moduli than those produced by Ht (Table 3.11). This suggests that although similar types of network were formed (weak viscoelastic gels) by the different treatments, the HP gels were 'stronger' and had greater elasticity than those of the equivalent Ht tofu.

It was observed that the HP tofu gels showed a slight increase in the frequency dependence of their moduli when compared to the equivalent Ht materials. This was accompanied by an increase in the tan δ for the HP tofu materials (Table 3.11). Such behaviour whilst indicative of increased 'consistency' suggests that the pressure induced material may represent a more 'liquid' system (increased contribution from the loss modulus (Ferry, 1980)). All of the gels showed some structural break down at higher frequencies, especially in the Ht tofu samples. Again the onset of such behaviour is indicative of the underlying structures present with the more 'rigid gel like' heat induced material showing damage at slightly 'earlier' frequency values. This frequency induced damage is indicative of increased interaction due to changes in the 'time scale' of the interactions present in a more structured systems (Ferry, 1980) and is unlikely to be related to any strain damage as the controlled stress instrument actually generates lower values at the higher frequencies.

Apichartsrangoon (2003) found that soy protein exhibited some structural modifications through the use of various pressure/temperature/time regimes (200 to 800 MPa at 20 and 60°C for 20 and 50 min) and the temperature caused rheological modification more than did pressure.

Okamoto *et al*. (1990) reported that the hardest SPI gels were obtained from the treatment of 400 MPa and 25°C for 30 min, however, pressure induced gels were softer than the heat (100°C for 10 min) induced gel.

Molina and Ledward (2003) stated that the structure of heat-set soy protein gels would be primarily maintained by disulphide bonds and hydrophobic interactions. On the other hand, pressure treatment denatured or unfold protein is due primarily to the rupture of electrostatic and hydrophobic linkages, since hydrogen bonds are essentially stable under such circumstances (Galazka and Ledward, 1998; cited in Molina and Ledward, 2003). Linkages, however, may well form, involving disulphide groupings and hydrogen bonds and, on subsequent release of pressure, additional hydrophobic and electrostatic linkages may also be set up (Molina and Ledward, 2003). Molina *et al*. (2002) also reported that the gel network formation resulted from HP treatment of the 11S protein being involved in SH/SS interchange. The –SH residues of the polypeptide chains were exposed during pressure treatment and in the presence of oxygen after release, they interacted to form intra- or intermolecular stable S–S bonds that help in the formation of the subsequent gel matrix.

Shen *et al*. (1991) revealed that GDL was soluble in water and $CaSO_4$ was suspended in water. Thus, the GDL solution mixed better with soymilk than the $CaSO_4$ suspension, and, therefore, the protein may have coagulated more completely in the case of GDL tofu.

3.3.3 Confocal scanning laser microscopy (CSLM) of heat induced and high pressure induced tofu

Figures 3.15, 3.16, and 3.17 show confocal microscopic plates of Ht and HP tofu gels made using different coagulants. The samples were stained for both fat material (red) and protein materials (green). The black area represent mainly of voids.

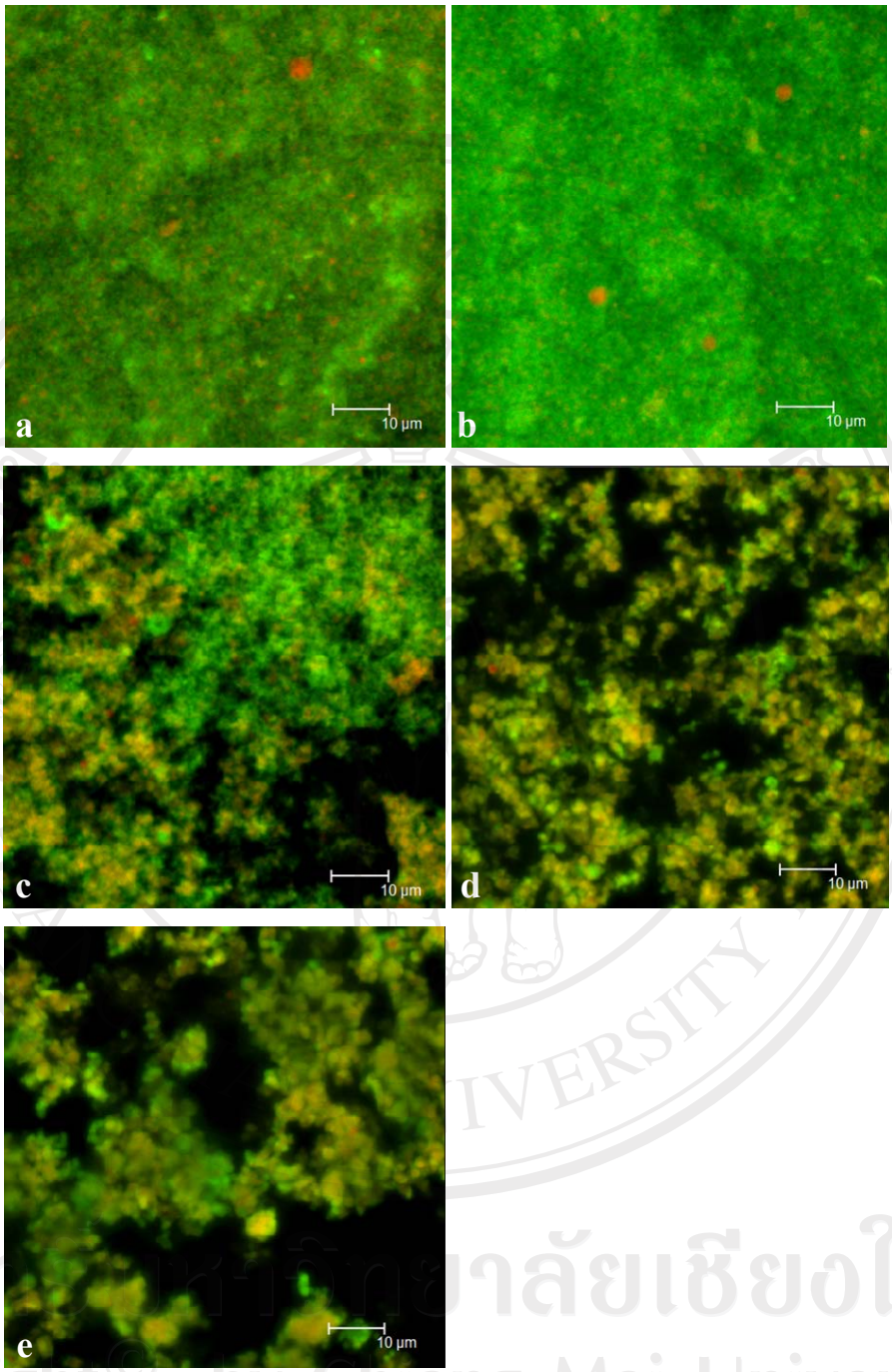

Figure 3.15 Typical confocal microscopy images of heat (Ht) induced GDL tofu and high pressure (400 or 600 MPa and 20°C for 10 min) (HP) induced GDL tofu processed using the following conditions; (a) Ht 0.2% w/v GDL; (b) Ht 0.4% w/v GDL; (c) HP 0.2% w/v GDL, 400 MPa; (d) HP 0.4% w/v GDL, 400 MPa; (e) HP 0.4% w/v GDL, 600 MPa.

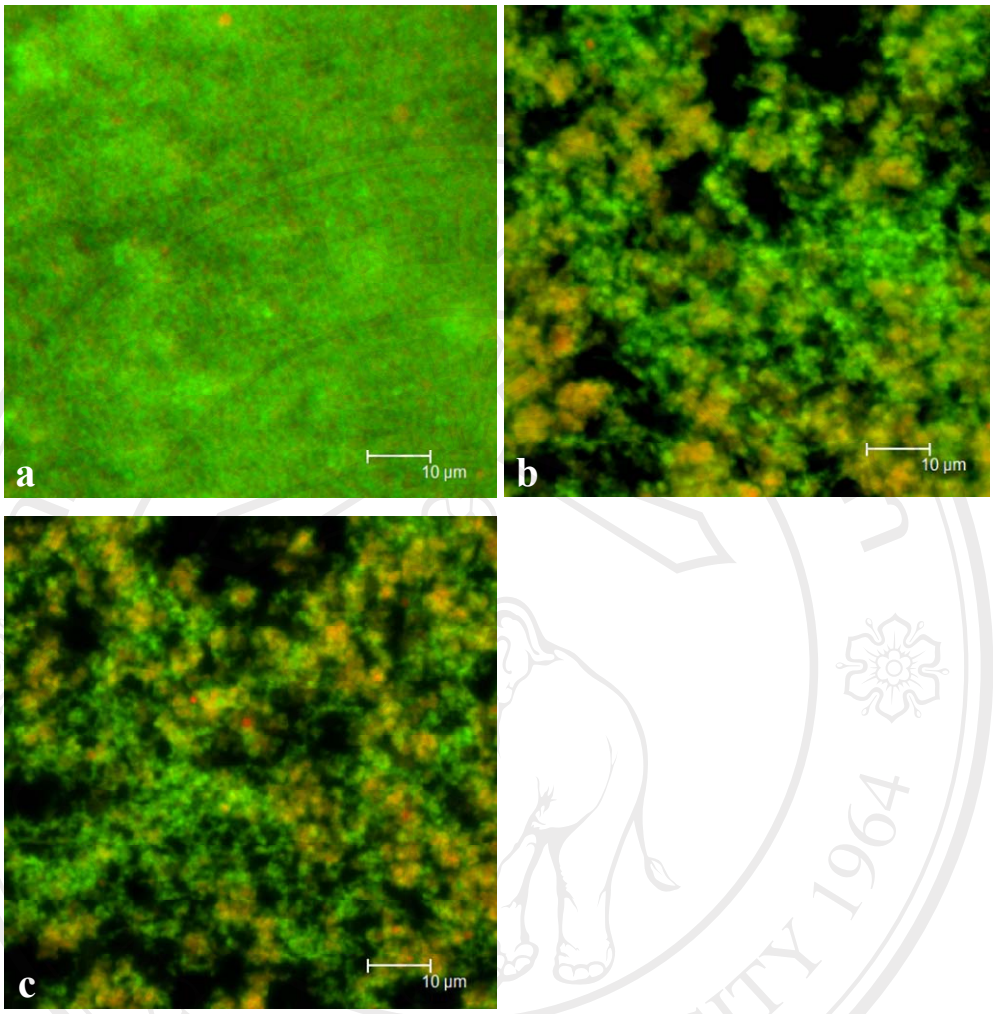

Figure 3.16 Typical confocal microscopy images of heat (Ht) induced and high pressure (400 or 600 MPa and 20°C for 10 min) (HP) induced tofu gels with added 0.4% w/v $CaSO_4.2H_2O$: (a) Ht tofu; (b) HP (400 MPa) tofu; (c) HP (600 MPa) tofu.

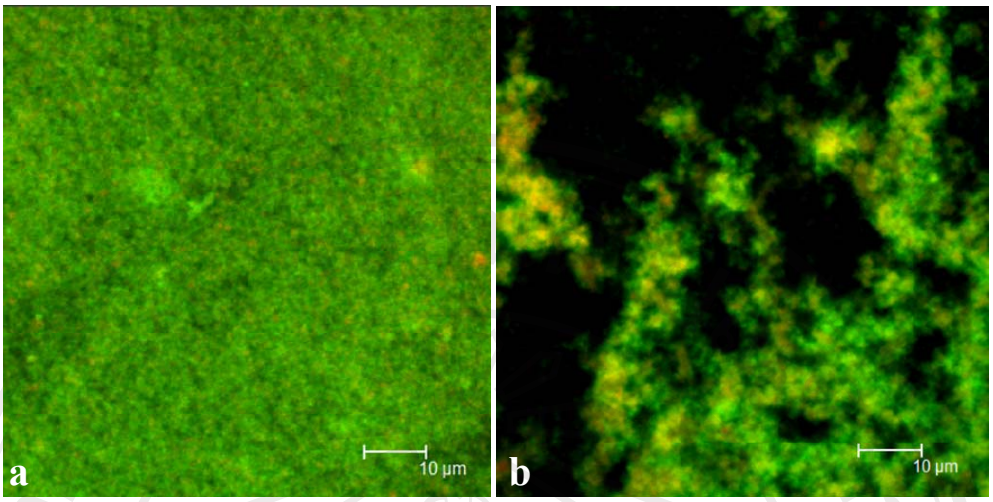

Figure 3.17 Typical confocal microscopy images of heat (Ht) induced and high pressure (400 MPa and 20°C for 10 min) (HP) induced tofu gels with added 0.14% w/v $CaCl_2.2H_2O$: (a) Ht tofu; (b) HP tofu.

The Ht tofu with added GDL (Figure 3.15a and b); added $CaSO_4.2H_2O$ (Figure 3.16a); and added $CaCl_2.2H_2O$ (Figure 3.17a) show an essentially homogenous spread of both the protein and the associated fat material. While there was some indication of variations in the overall material density, there was no evidence of any open 'voids' in the structure. The structure of the HP tofu with added GDL (Figure 3.15c, d and e); added $CaSO_4.2H_2O$ (Figure 3.16b and c); and added $CaCl_2.2H_2O$ (Figure 3.17b) contrast sharply with those of the Ht samples in that while the fat material again still seems to be associated with the protein material the overall structure seems to be more 'aggregated' producing a loose 'network' of material containing a considerable number of, presumably, liquid filled spaces. Such 'gels' showed considerable syneresis when processed (cut, milled and so on).

The confocal microscopy images supported the rheological results (section 3.3.2.3) in that the loss tangents of HP tofu gels were higher than those of the equivalent Ht tofu gels because the HP gels contained more voids than the Ht gels and the strands of the HP gels were thicker than those of the Ht gels, indicating HP gels had strong but looser structure than the Ht gels.

These results were also supported by the subsequent studies on the water holding capacity of the various tofu samples (section 3.3.4) which showed that the amounts of water released from HP tofu were higher than those from the equivalent Ht tofu.

The results obtained from the HP samples suggest that a 'coarse' open network is formed, having poor reworking and water holding properties. It has been proposed that this type of structure occurs when the aggregation of coagulum occurs too quickly, compared to the time scale of the denaturation of the protein, so that a random network, that is unable to hold water, is formed and syneresis occurs in the subsequent product (Kinsella *et al*., 1994). If the aggregation process is slower relative to the protein denaturation an ordered structure will be promoted, allowing the denatured molecules to orient themselves in a systematic fashion, prior to aggregation (Hermansson, 1978), as appears to occur on heat treatment.

3.3.4 Water holding capacity of heat processed and high pressure induced tofu

Water holding capacity (WHC) is the ability to physically hold water against gravity. This is related to viscosity of food system and is influenced by pH, ionic strength and temperature (Kinsella, 1979). Figure 3.16 shows WHC of Ht 0.4% GDL tofu, Ht 0.4% $CaSO_4.2H_2O$ tofu, HP 0.4% GDL tofu, and HP 0.4% $CaSO_4.2H_2O$ tofu, as water released per gram sample.

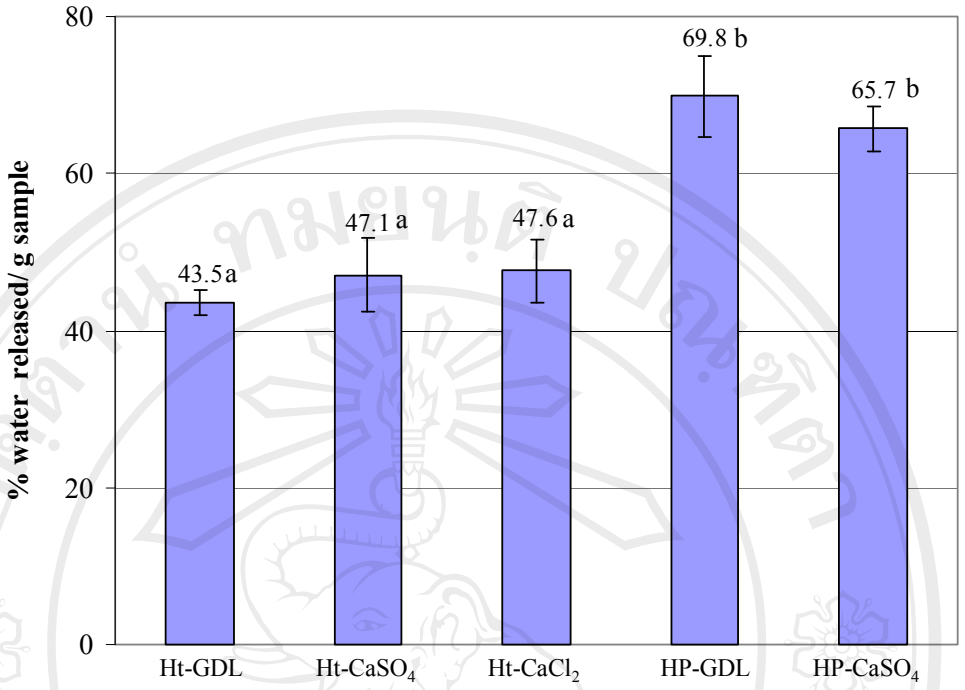

Figure 3.18 Water holding capacity of heat (Ht) processed and high pressure (400 MPa 20°C 10 min) (HP) induced GDL and $CaSO_4.2H_2O$ tofu gels (mean of 6 replications). Means followed by the same letters (a, b) are not significantly different ($p<0.05$).

Results show water released from the HP tofu samples are higher that than those from the Ht processed tofu materials with the addition of both GDL and $CaSO_4.2H_2O$ ($p<0.05$). Significant differences were not observed between gels obtained from the same heat or high pressure treatment, WHC of the Ht GDL tofu was not significantly different from the Ht $CaSO_4.2H_2O$ tofu. The WHC of the HP GDL tofu was not significantly different from the HP $CaSO_4.2H_2O$ tofu. This result suggested, and was corroborated by images from the confocal microscopy, that HP gels were less homogenous than equivalent Ht gels.

The results were consistent with those of Puppo and Anon (1998) who reported that a protein gels with homogeneous fine structures gave high water retention ability, when compared to the gels with nonhomogeneous structures. These

had a high degree of syneresis. The work of Pares *et al*. (1998) also supported that the loss of homogeneity of the structure, the 'crack' or 'empty' or void space, could explain the reduction in water retention of the gels. Kao *et al*. (2003) also indicated that with the optimum coagulant concentration, a uniform, continuous tofu network was formed, and it was able to trap more water and soluble substances.

Tay and Perera (2004) reported that soft gel with higher proportion of 7S:11S and incubated for 20 min had a poorer ability to hold water than the harder gels incubated for the same period of time. They explained that the softer gels may have fewer networks to trap water molecules than harder gel. But at the incubation time increased from 40 to 60 min, the WHC of the soy gels containing a higher proportion of 11S was lower than those containing higher proportion of 7S. A possible explanation could be that when the duration of heating increased, the gap in the network of gels containing a high proportion of 11S could 'shrink' and thus entrap less amount of water. The network of gels containing a higher proportion of 7S could shrink less.

3.3.5 Gel electrophoregrams of heat processed and high pressure induced tofu : native polyacrylamide gel electrophoresis (native PAGE)

The native PAGE was carried out without adding SDS and a reducing reagent. The samples were not heated before application. Therefore, the native PAGE patterns can detect the heat and pressure effects on the soy protein subunits without interference from SDS or reducing agent.

Figure 3.19 shows the typical native PAGE patterns of heat (Ht) induced and high pressure (HP) induced tofu samples with 0.4% w/v added GDL, 0.4% w/v added $CaSO_4.2H_2O$, and 0.14% w/v added $CaCl_2.2H_2O$. It clearly indicates some changing of the bands of heat treated and high pressure treated samples from those of the raw soymilk.

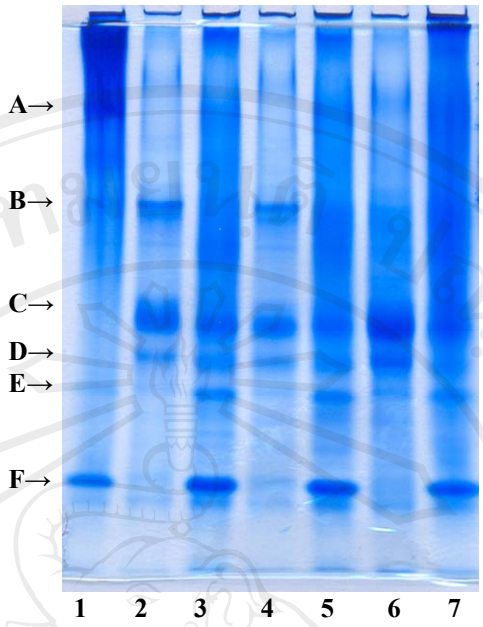

Figure 3.19 Typical native PAGE patterns of heat (Ht) and high pressure (400 MPa 20°C 10 min) (HP) induced tofu with added 0.4% w/v GDL, 0.4% w/v $CaSO_4.2H_2O$, and 0.14% (w/v) $CaCl_2.2\ H_2O$: lane 1, raw soymilk (control); lane 2, Ht GDL tofu; lane 3, HP GDL tofu; lane 4, Ht $CaSO_4$ tofu; lane 5, HP $CaSO_4$ tofu; lane 6, Ht $CaCl_2$ tofu; lane 7, HP $CaCl_2$ tofu.

The raw soymilk (lane 1) as a control sample showed that not many protein bands appear in the Native PAGE pattern. It is possibly that the large molecular weight soy proteins such as the 11S fraction (around 355 kDa) and 15S fraction (around 600 kDa) were too large to pass into the gel pore structure of a 7.5% T gel. Moreover, there are not only protein molecules in the raw soymilk but also other components including large molecules of both fat and carbohydrate materials that might block the protein molecules trying to pass into the gel.

Tofu samples (lane 2 to lane 7) show more protein bands than in the raw soymilk (lane 1). The Ht samples with GDL, $CaSO_4$, and $CaCl_2$ (lane 2, 4, and 6, respectively) show essentially the same pattern of protein bands because each sample

has been processed under the same heat treatment conditions, preheated soymilk at 97-100°C for 7 min followed by coagulation at 70°C for 60 min. The results suggested that tofu prepared with the difference coagulants show no difference when compared to the soluble protein profiles of heat treated soy proteins.

Similarly, the high pressure induced samples with added GDL, $CaSO_4.2H_2O$, and $CaCl_2.2H_2O$ (lane 3, 5, and 7, respectively) show the same patterns of protein bands because each sample was processed under the same high pressure conditions (400 MPa 20°C for 10 min). The results indicated that tofu prepared from different coagulants showed no difference in the soluble protein profile in any of the pressurised soy protein samples.

Band A appears in raw soymilk near the top of the gel but disappears in the Ht and HP samples. Band B, C and D in the middle of the gel appear in both the Ht and HP samples. Band E, that appears in the HP samples is less intense in both the raw soymilk and the Ht samples. These results indicated that proteolysis occurred in both the Ht and HP samples. Soy proteins in soymilk were denatured and dissociated into subunits by heat or high pressure.

The appearance of band F and the remained of some large molecules on the top of the PAGE gel in the lanes 1 (raw soymilk) and lane 3, 5 and 7(HP samples) indicated that the heat treatment affected protein denaturation stronger than the high pressure did.

Figure 3.20 and Figure 3.21 depict the native PAGE patterns of both heat induced and high pressure induced GDL tofu and $CaSO_4$ tofu samples respectively. Both show that new bands appeared in Ht and HP samples at about 66 kDa.

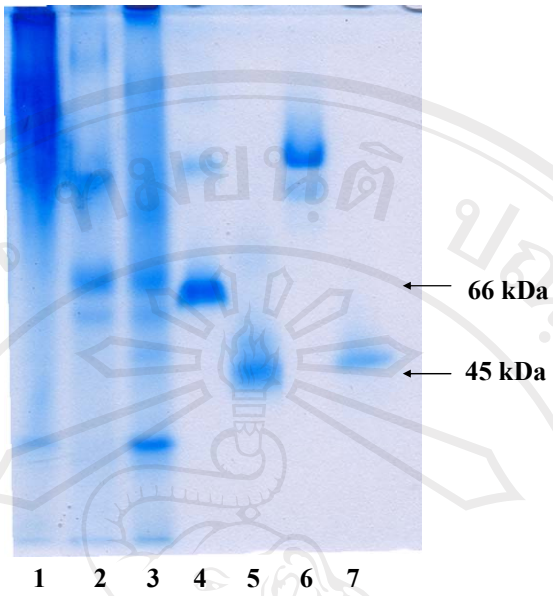

Figure 3.20 Typical native PAGE patterns of heat (Ht) and high pressure (400 MPa 20°C 10 min) (HP) induced tofu with added 0.4% w/v GDL: lane 1, raw soymilk (control); lane 2, Ht GDL tofu; lane 3, HP GDL tofu; lane 4-7, standards.

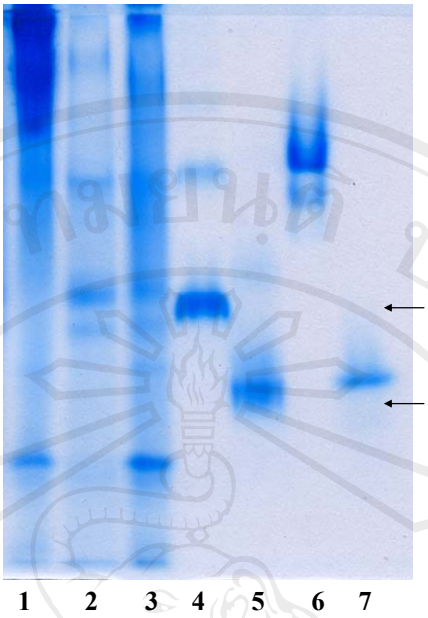

Figure 3.21 Typical native PAGE patterns of heat (Ht) and high pressure (400 MPa 20°C 10 min) (HP) induced tofu samples with added 0.4% w/v $CaSO_4.2H_2O$: lane 1, raw soymilk (control); lane 2, Ht $CaSO_4.2H_2O$ tofu; lane 3, HP $CaSO_4.2H_2O$ tofu; lane 4-7, standards.

Apichartsrangoon (2002) reported that the formation of covalent disulphide cross-link was found in the heat treated soy protein gels and heated treated gluten gels However, Kohyama *et al*. (1995) considered that the formation of disulphide bonds is very slow at the low pH region, and the contribution of disulphide bonds on the heat induced tofu is negligible. Saio *et al*. (1971) indicated that intermolecular S-S bonds were involved in the protein network of the 11S globulin but not of 7S globulin due to the fact that the 7S globulin has a very low sulphur content and disulphide bridges do not appear in the binding between subunits (Koshiyama, 1971). However disulphide bonds appear to participate in the binding between pairs of acidic and basic subunits of the 11S globulin, which contains 48 sulphur atoms (Kitamura *et al*., 1976).

Puppo *et al*. (2004) revealed that the SPI (pH 8) is constituted by native β-

conglycinin and glycinin. High pressure treatment at higher than 200 MPa produced partial unfolding of the 7S and 11S fractions, and aggregation of the protein the 11S fraction, yielding large aggregates that could not enter the PAGE gel. The aggregates were stabilized by disulphide bridges and probably noncovalent bonds.

Apichartsrangoon (2003) reported that electrophoregram of pressurized soy protein at 400 to 800 MPa at 60°C displayed some slight reduction in the intensity of some bands, especially in the low molecular mass region (14.4 kDa). Soy glycinin could be dissociated into subunits after high pressure 300 MPa or over and more sulphydryl groups as well as hydrophilic and amino acid residues had been reported (Zhang *et al*., 2003).

3.4 Conclusions

The present results suggest that tofu gels can be formed by pressurised raw soymilk with added coagulants such as GDL and $CaSO_4.2H_2O$; these products differ significantly from those traditionally produced by heat treatment. The gels produced by pressure, although having a higher overall 'consistency' (increased moduli values) seem essentially to be more a 'liquid' viscoelastic system (increase in tan δ) than those obtained by heat treatment. The differing structures produced can subsequently be observed using microscopic techniques. The more 'open' structure produced by the pressure processing contains considerable 'void' areas which are absent in the more homogeneous heat treated material (Figure 3.15, and 3.16). Such differences may well have considerable implications in terms of subsequent product development. While such processing may give rise to differing systems/structures with possibly new or modified organoleptic properties, the more open structure (and associated syneresis), could cause considerable problems if further reworking of the material is required.