CHAPTER 2

LITERATURE REVIEW

2.1 Tofu

2.1.1 General

Tofu is a water-extracted and salt- or acid-coagulated soy protein gel, with water, soy lipids, and other components trapped within the network. Depending on the method of preparation used, the textural properties and composition of commercial tofus may vary. They are generally classified as dry tofu (Doufugan), firm tofu (Momen), soft tofu, silken tofu (Kinu), and filled (packed) tofu (Liu and Chang, 2004).

In the traditional method of preparing tofu, soybeans are washed, soaked, and then ground with added water. The resultant slurry is then filtered. This filtrate is collected to become a raw soymilk. The milk is then heated before a coagulant is subsequently added to form a curd or tofu. The 'firm' and 'soft' tofus are made by pressing after coagulation to remove any excess whey, but the 'silken' and 'filled' tofus are made without the removal of the whey after coagulation (Saio, 1979; Fukushima, 1981; Shen *et al*., 1991; Kohyama *et al*., 1992; Shih *et al.,* 1997; Liu and Chang, 2003; Liu and Chang, 2004; Cheng *et al*., 2005). Therefore, silken tofu and filled tofu have the highest water content and soft.

Complex interactions of many contributing factors occur in the making of tofu. The processing methods play a major roles on the tofu yield, moisture content, hardness and elasticity of the end products (Cai *et al*., 1997). In practice increasing the soymilk concentration gives a 'harder' tofu (Cheng *et al*., 2005).

2.1.2 Factors affecting tofu manufacture

The quality of tofu is influenced by the quality of soymilk and its subsequent coagulation. The quality of soymilk depends on the variety of soybeans used and the conditions of soymilk preparation. The coagulation process depends on the

concentration and temperature of soymilk, the type and relative amount of coagulant, and the mixing method and time (Liu, 1999). The protein content in the raw soybean material is usually considered to be the most important factor in determining the quality of soymilk extracted and acceptability of the final tofu produced The correlation between the protein content of the soybeans and soymilks and between the content of soybean protein and the total solid content of soymilk are significant ($p<0.05$) (Min *et al*., 2005). The hardness of 'filled' tofu increased with protein content in soymilk (Shen *et al*., 1991). Soymilk proteins that are richer in sulphydryl (SH) groups have been shown to yield a firmer tofu product (Obata and Matsuura, 1993).

The amount and type of coagulant added to soymilk are critical factors in tofu making as they particularly change the textural properties of the tofu produced. The amount of coagulant required is positively correlated with the phytate content, pH, and 7S protein content but negatively correlated with the overall protein content, 11S protein content, 11S/7S ratio, titratable acidity and calcium content in the original soymilk. Within the same soymilk material, higher protein levels required the use of more coagulant, however a higher protein concentration during heating resulted in less coagulant being required by each gram of protein during coagulation. Less coagulant was required when soymilk heating time was increased, and more coagulant was required when soymilk was heated more quickly. Different sequences of heating and dilution of the protein has resulted in different coagulant requirements (Liu and Chang, 2004). When a suitable amount of coagulant is used, the whey becomes transparent, with an amber or pale yellow colour and a sweet taste. There should remain no uncoagulated soymilk protein in the whey.

Coagulation temperature affects coagulation. At higher temperatures, the proteins possess a high energy state, which leads to a faster coagulation. The resulting tofu tends to have small network with reduced water holding capacity, hard texture, and therefore, a low bulk yield. When the coagulation temperature is low, the effect is the opposite. However, if the temperature is too low, coagulation is incomplete and the resulting tofu contains too much water, and is too soft to retain its shape. Therefore, the selection of the temperature during tofu production depends on the type

and concentration of coagulants, the mode of adding coagulants, and the type of tofu to be made (Liu, 1999).

Coagulants used for tofu making vary widely. They include calcium sulphate, various chlorides (calcium chloride, magnesium chloride) and glucono-δ-lactone (GDL) (Liu, 1999). Other coagulants have been reported to make a 'filled' tofu, including natural bittern, magnesium sulphate, calcium primary phosphate, calcium lactate, and transglutaminase (Matsuura *et al*., 2000).

Calcium sulphate ($CaSO_4$) is a naturally occurring mineral, also known as Plaster of Paris, or gypsum. Calcium sulphate is used to provide a source of calcium ions. It is a generally permitted additive under Directive 95/2/EC (Emerton, 2003). Calcium sulphate is reported to be used mainly in the making of 'pressed' tofu (Tsai *et al*, 1981; Sun and Breene, 1991; Cai and Chang, 1998; Min, *et al*., 2005). However, Liu (1999) stated that calcium sulphate can be used to make any type of tofu. Because of its high ability to incorporate water into tofu, the calcium sulphate coagulant gives higher bulk yields than the equivalent chloride-types. However, tofu made from calcium sulphate has slightly inferior flavour when compared with those made from chloride-type coagulants. Calcium sulphate forms an unstable suspension when mixed with water because of its limited solubility. This factor makes calcium sulphate difficult to mix well into the soymilk, resulting in tofu with a less consistent texture. In addition, the reactivity (or coagulation potency) of calcium sulphate decreases gradually during storage, particularly in the presence of moisture. Therefore, calcium sulphate should be mixed with water just before use (Liu, 1999).

Chloride-type coagulants are more water soluble than those based on calcium sulphate, and tend to coagulate the soybean protein faster. Tofu made with chloride-type coagulants was not as soft and smooth as those made with calcium sulphate. Therefore, chloride-type coagulants are not suitable for making 'silken' tofu. Because such coagulants do not incorporate as much water as others, they tend to give an overall lower bulk yield. The optimum concentration level of chloride-type coagulants is very limited and the coagulation take place rapidly, so concentration range of calcium chloride that gave optimum texture for tofu is narrower than that of

calcium sulphate (Saio, 1979; Liu, 1999). Calcium chloride is a generally permitted additive under Directive 95/2/EC (Emerton, 2003).

GDL is a neutral cyclic ester of gluconic acid, produced by the fermentation of glucose or glucose containing raw materials such as glucose syrup. GDL is completely metabolized in the body in the same way as any carbohydrate. Under Directive 95/2/EC, it is generally permitted additive (Emerton, 2003). GDL is particularly suitable for large-scale production of 'silken' tofu (Liu, 1999) and has been widely used to make 'filled' tofu with a good texture and an extended shelf life (Guo and Ono, 2005). However, it is not suitable for making 'firm' tofu (Tsai *et al*., 1981).

The great advantage of GDL as a tofu coagulant over the other coagulants is that a small amount of GDL can be mixed with the cold soymilk and the milk is then poured into containers, which are sealed immediately. Upon immersing the sealed container in hot water, coagulation begins and proceeds slowly as a result of heat-activated hydrolysis of lactone into gluconic acid (Liu, 1999, Schwertfeger and Buchheinm, 1999). Under pressure, the hydrolysis of GDL could be accelerated as compared with reaction under ambient pressure (Schwertfeger and Buchheinm, 1999).

Generally in the tofu making, soymilk must be properly heated before adding coagulants. This heat treatment is essential not only for improving nutritional quality and reducing the 'beany' flavour but also for denaturing the proteins so that they can coagulate into curd in the presence of the coagulant. Variations in the temperature and time of heating soymilk before adding coagulant to make tofu have been reported in the literature. This includes heating the milk to 100°C and then cooling (Berk, 1992), heating to 100°C for 3 min (Fukushima, 1981), heating to 90°C and held for 5 min (Kao *et al*., 2004), heating for about 3 min after boiling (Saio, 1979), heating to 130°C for at least 1 sec (Matsuura, 1985), and heating to 85°C for at least 5 min (Weaver *et al*., 2002).

2.1.3 Tofu gelation mechanism

A two-step mechanism for the gelation of tofu has been proposed by Kohyama *et al.* (1995) as shown in Figure 2.1. The first step is the heat-induced denaturation of the soy protein which exposes the hydrophobic regions of the protein molecule to the outside. Such denatured soy protein is negatively charged (Kohyama and Nishinari, 1993) and the change in ionic conditions induced by GDL or calcium ions neutralises the net charge on the protein. As a result, the hydrophobic interactions of the neutralised proteins becomes more predominant and induces their random aggregation, leading to gel formation (deMan *et al.*, 1986). Shun-Tang *et al*. (1999) added that the lipids were incorporated into the coagulum by conjugating with the protein particle during the soymilk gelation.

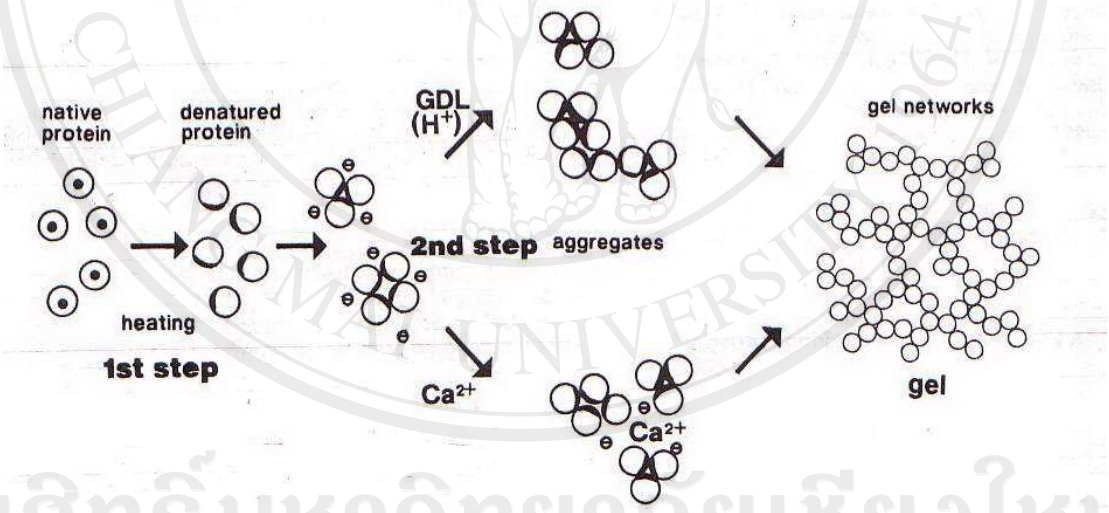

Figure 2.1 Gelation mechanism of soybean protein in the presence of GDL or calcium sulphate (from Kohyama *et al.*, 1995).

The unique textures of tofu are believed to be as a result of both the intermolecular interchange between the exposed -SH and S-S groups and the intermolecular hydrophobic interactions among the exposed hydrophobic amino acid residues of the denatured protein (Fukushima, 1991).

Ono *et al*. (1993**)** reported that addition of calcium sulphate to make tofu means that, the calcium can bind to the proteins in soymilk through the carboxyl groups of the glutamyl and asparagyl residues. It is assumed that one of the binding site is the imidazole groups in the histidine residues of the proteins. When calcium was added to soymilk, it should bind to phosphate groups of phytate and to carboxyl and imidazloe groups of the proteins, and the pH would then decrease gradually. It is considered that the pH decrease upon the addition of calcium is mainly due to the production of calcium phytate. The decrease in solubility of the protein by calcium addition was accompanied by an associated pH decrease. The addition of calcium can produce tofu curd at a higher pH than that produced without the addition of calcium.

Kohyama *et al*. (1995) found that the structures of calcium induced gels inferred that the rheological properties of tofu were quite similar to those of GDL gels. The difference between GDL-induced and calcium-induced gelation, however, was mainly related to the reaction rate, that is, gelation by calcium ions was shown to be faster. The addition of GDL or calcium induced gelation by promoting the aggregation of the proteins via hydrophobic interactions.

Saio (1979) and Cheng *et al*. (2005) reported that GDL induced tofus showed higher breaking stresses than the calcium induced tofus. Saio (1979) also stated that the network of GDL tofu consisted of flocculent aggregates while that of calcium tofu showed a spongy structure with tight frames.

2.2 Soybean proteins

2.2.1 General composition

Soybean contains about 40% protein, 20% oil, 35% carbohydrate, and 5% ash of total dry matter. Other valuable components found in soybean include phospholipids, vitamins, and minerals. Furthermore, soybeans contain many minor substances, some of which, such as trypsin inhibitor, phytates and oligosaccharides are known to be biologically active. Other components, such as isoflavones are being recognised for their powerful ability to prevent human cancers and other diseases (Liu, 1999).

The greatest component presented in soybean is protein which contains all of essential amino acids required for human or animal nutrition like proteins of most other leguminous plants, soy protein is low in sulphur containing amino acids such as methionine, cystine, and threonine. However, soybean contains sufficient lysine, which is deficient in most cereal proteins (Liu, 1999). The acidic amino acids (glutamine and aspartic acid) constitute approximately 25% of the total amino acid present in soybean, whilst, the basic amino acids (lysine, arginine, and histidine), constitute one-fifth. The amino acids with hydrophobic side chains (glycine, alanine, valine, leucine, and isoleucine) account for a further 19-20% of the total protein compared to the mean values of 9.1-9.8% for total aromatic amino acids (phenylalanine, tryptophan, and tyrosine) (Zarkadas *et al*., 1993).

2.2.2 Soy proteins; classification and nomenclature

Soy proteins have been classified in many ways. Based on biological function in plants, the majority of soybean proteins are considered storage proteins. Based on solubility patterns, most soy protein is globulins which are soluble in salt solutions. The two main type of storage proteins in soybeans are β-conglycinin and glycinin which account for 65-80% of total seed proteins (Liu, 1999).

On the basis of their sedimentation constants at pH 7.6 and ionic strength buffer of 0.5, the globulins are characterized as 11S or glycinin and 7S or β- and γ-conglycinin (Fukushima, 1991), with other less abundant globulins including 2S or α-conglycinin, 9S globulins, and 15S globulins (Estrada-Giron *et al*., 2005). S stands for Svedburg unit. It is computed as the rate of sedimentation per unit field of centrifugal strength based on the following equation:

$$S = (dc/dt)\ \omega^2 c$$

Where, c denotes the distance from the centre of the centrifuge, t is time, and ω is angular velocity. The value for S ranges between 1 and 200, with a unit of 10^{-13} sec (Liu, 1999).

The 11S and 15S fractions are pure proteins. More specifically, the 11S fraction is the soybean glycinin and accounts for at least 33% of extractable protein,

whereas the 15S fraction is though to be a polymer of glycinin and accounts for about 10% of extractable protein. In contrast, the 2S and 7S fractions are heterogeneous. The 2S fraction accounts for 20% of the extractable protein and includes the Kuniz and Bowman-Birk trypsin inhibitors and cytochrome C. The 7S fraction accounts for an additional third of the extractable protein and consists of conglycinin, α-amylase, lipoxygenase, and hemagglutinin (lectin) (Liu, 1999).

The differences in composition and structure between the two major soybean globulins, β-conglycinin (7S) and glycinin (11S), are exhibited by differences in both nutritional and functional properties (Riblett *et al*., 2001). The glycinin usually contains three to four times more methionine and cysteine per unit protein than β-conglycinin. The two globulins also show considerable differences in key functional properties, including gel-making ability, thermal stability, and emulsifying capacity (Liu, 1999; Renkema *et al*., 2001; Yamauchi *et al*., 1991).

2.2.2.1 β-conglycinin

β-conglycinin is the major component of the 7S fraction. It shows denaturation temperature at pH 7 of 67°C (Hermansson, 1978) and precipitates at a pH between 4.0 and 5.6 (Liu, 1999). The average β-conglycinin content was found to be 18.5% as a seed protein (Murphy and Resurreccion, 1984).

β-conglycinin (7S globulin) is a trimeric glycoproteins with a mloecular weight (MW) of about 180 kilo daltons (kDa). It has three major types of subunits, designated as α′, α, and β with mloecular weights were estimated at 57, 57, and 42 kDa, respectively. All three major subunits are rich in aspirate/asparagines, glutamate/glutamine, leucine and arginine but contain small amounts of cysteine and methionine (Liu, 1999).

2.2.2.2 Glycinin

Glycinin (11S fraction) is the purified from the 11S globulin. It shows a denaturation temperature at pH 7 of 80°C (Hermansson, 1978) and precipitates at a pH between 4.4 and 6.8 (Liu, 1999). Glycinin is the largest single fraction of total soy

seed protein, and its overall content averaged some 51.0% by weight of the total protein assayed (Murphy and Resurreccion, 1984).

The model of glycinin is hexameric with a MW of about 360 kDa. Its monomeric subunits have the generalized structure A-S-S-B, where A represents an acidic polypeptide of 33-34 kDa; B is a basic polypeptide of about 20 kDa; and S-S is a single disulfide bond that links the two polypeptides (Liu, 1999).

Abbott *et al*. (1996) reported secondary structure of glycinin as approximately 33% β structure, 25% α-helics, 31% turns, and 12% unordered. Their data indicated that glycinin has the same secondary structure in solution and in the hydrate solid state. Nothing is known about the tertiary structure of glycinin (Liu, 1999). However glycinin has a complex quaternary structure consisting of two layers of trimers. Each trimer has three acidic and three basic polypeptides paired and held together by disulphide and hydrogen bonds, with basic and acidic peptides alternating (Badley *et al*., 1975). These bonds can be disrupted by urea, strong acid, strong base, heat, or sodium dodecylsulphate in combination with a disulfide reducing agent. As a result, the quaternary structure can be altered (Liu, 1999).

2.2.3 Soy protein gelation

Gelation is a functional property of proteins related to protein structure and their rheological characteristics (Moure *et al*., 2006). Protein gel is made up of polymers cross linked via either covalent or noncovalent bonds to form a network that is capable of entrapping water and other low-molecular-weight substances (Damodaran, 1996). Clark *et al*. (2001) suggested that homogeneous heat set globular protein gelation proceeds in three main stages, i.e., an unfolding step, a step of linear fibrillar aggregation, and a step of random association of the fibrils.

The major soy proteins glycinin and conglycinin both have the ability to form an ordered gel structure. These proteins have complex quaternary structures that easily undergo association-dissociation reactions, depending on environmental conditions. Several environmental factors have been reported to influence the gelation

of soy proteins including; protein concentration, temperature, pH, salt, enzyme, pressure level, and the presence of additives (see earlier).

The gelation phenomena of heat induced soy proteins was proposed as shown in Figure 2.2. When heated, the soy protein dispersion (sol) increases in viscosity and undergoes an irreversible change to the pro-gel state. Pro-gel becomes a gel when it is cooled, and viscosity increases. This step is irreversible. However, excessive heating irreversibly converts the gel or pro-gel into metasol, which does not gel at all due to thermal degradation of the proteins (Yamauchi *et al*., 1991).

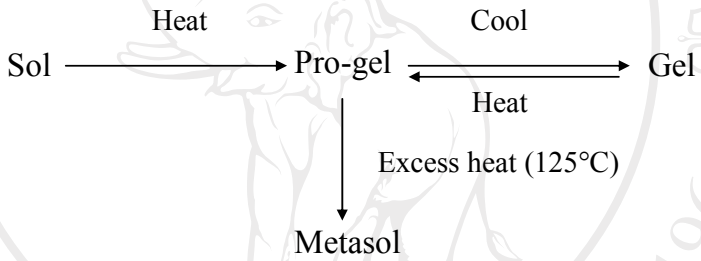

Figure 2.2 Phenomena of heat induced soy proteins gelation (from Oakenfull *et al*., 1997).

Utsumi and Kinsella (1985) indicated that in heat induced gels, the basic subunit of 11S globulin interacted with 7S globulin in soy isolate gels. The glycinin strands were more regular and the degree of cross linking lower than in the case of conglycinin-rich gels. The aggregated structure of glycinin that formed at 85°C in the presence of salt was interpreted as a transient state corresponding to the soluble aggregate found in early stage of heating of a dilute glycinin solution (Hermansson, 1986). When gels were made by heating at 80°C for 30 min the 7S gels were harder than the equivalent 11S gels (Utsumi and Kinsella, 1985).

The aggregation profiles of 11S and 7S proteins with GDL at room temperature showed that 11S protein formed large aggregates somewhat faster than 7S

protein (Tay *et al*., 2005). The faster aggregation leads to faster overall gelation of the system (Kohyama *et al*., 1995).

The soymilk from soybean which had high protein particle content formed harder tofu curds (Guo and Ono, 2005). Riblett *et al*. (2001) reported that the gel forming properties of β-conglycinin and glycinin among four soybean genotypes showed that the coagulants prepared from the materials with higher lysine contents gave stronger, firmer, more elastic gels.

Soy protein gels can be formed by high pressure treatment. The nature of high pressure induced gels will be very different to those induced by heat, since heat treatment will primary affect hydrogen bonded networks while pressure will more effectively disrupt hydrophobic and electrostatic interactions (Ledward, 1995). The reviews of high pressure induce soy protein gels are given in section 2.3.4.

2.2.4 Trypsin inhibitors

Trypsin inhibitors (TI) are the major antinutritional factors present in soybeans. They occur in two forms: the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor (BBI). The KTI has MW between 20 and 25 kDa, with a specificity primarily directed toward trypsin. This inhibitor was shown to combine tightly with trypsin in a stonichiometric fashion, i.e., 1 mole of the inhibitor inactivates 1 mole of trypsin. The amino acid sequence of the inhibitor consists of 181 amino acid residues and two disulfide bonds, with a reactive site at the residues of Arginine63 and Isoleucine64 (Liu, 1999).

The BBI is a single polypeptide chain of some 71 amino acids including seven disulfide bonds (Birk, 1985). It MW is about 8 kDa. The BBI is capable of inhibiting both trypsin and chymotrypsin at differing reactive sites, one at lysine16-Serine17 against trypsin (Birk, 1985) and the other at leucine44-Serine45 against chymotrypsin (Liu, 1999). Wu and Sessa (1994) found that BBI has 61% β-sheet, 38% unrecorded form, 1% β-turn, and 0% α-helical form.

Trypsin inhibitors play a key role in the digestions of protein in animals (Liener, 1981). There have been many reports on the negative effects of trypsin

inhibitors and their subsequent nutritional implications for both animals and humans. An assumption is that soybean trypsin inhibitors can cause growth rate reduction in rat by reducing the digestibity of the proteins (Liu, 1999). Another significant finding was that trypsin inhibitors could cause hypertrophy of the pancreas in chicks (Liu, 1999). On the other hand, soybean BBI has been reported to have an anticarcinogenic effect in several tissue/organs (colon, liver, lung, oesophagus, and cheek pouch) of mice, rats, and hamsters (Kennedy, 1994; Kennedy, 1998).

Liener (1981) reviewed that the mode of action of the soybean inhibitor, finding that soybeans and the trypsin inhibitor itself could cause hypertrophy of the pancreas. This led to suggestion that the growth depression caused by the trypsin inhibitor might be the consequence of an endogenous loss of essential amino acids being secreted by a hyperactive pancreas. The pancreas secretion is controlled by a mechanism of feedback inhibition which depends upon the level of trypsin and chymotrypsin present at any given time in the small intestine. When the level of this enzyme falls below a certain critical threshold value, the pancreas is induced to produce more enzymes. The suppression of negative feedback inhibition can occur if the trypsin is complexed with the inhibitor or by dietary protein itself. It is believed that the mediating agent between trypsin and the pancreas is the hormone cholecystokinin (CCK), which is released from intestinal mucosa when the level of trypsin in the intestine fails below its threshold level. Those relationships are illustrated in Figure 2.3.

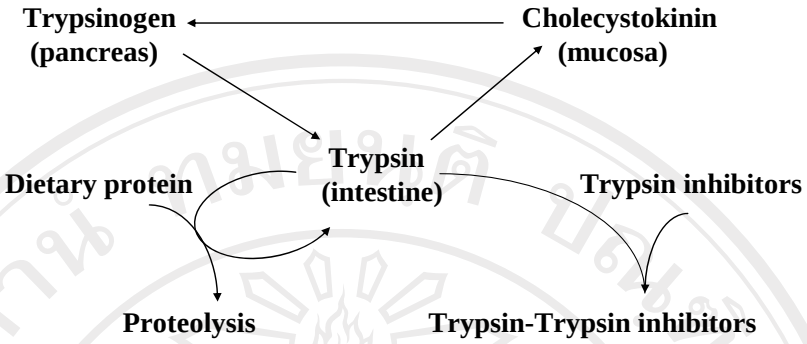

Figure 2.3 Regulation of the secretion of trypsin by the pancreas, cholecystokinin, and trypsin inhibitors (from Liener, 1981).

The extent of the destruction of trypsin inhibitors in soymilk for maximum nutritive value or protein efficiency ratio was reported to be about 80-90% (Hackler *et al*., 1965). A later work suggested that the time/temperature requirement be based on an 85% deactivation of the trypsin inhibitors (Wilson, 1989 cited in Liu, 1999).

Extensive efforts have been made to inactivate trypsin inhibitors from soybeans and soymilks. Most approaches have been based mainly on heat treatment. Soybean trypsin inhibitors are rather heat stable, long treatment times for example, 30 min at 100°C or 22 min at 110°C (Liu, 1999) or ultrahigh-temperature (UHT) of 143°C for 62 sec (Kwok *et al*., 2002) are required to reach 90% inactivation. Recently, van der Ven *et al*. (2005) reported that a 90% inactivation of trypsin inhibitor activity in soymilk with treatment time of less than 2 min can be reached at temperature between 77 and 90°C and at pressure between 750 and 525 MPa. Kwok *et al*. (2002) stated that vitamins, lysine, and taste in soymilk are stable during heat treated at 90°C for around 50 min.

2.3 Heat and pressure treatments

2.3.1 General

Heat is the most commonly used agent for food processing and preservation. It relies on the principle of microbial destruction and inactivation. Heat treatment causes a major change in the chemical and physical properties of the food components. High heat treatment will lead to protein denaturation, enzymatic inactivation and sometimes potential oxidation. This can cause a wide range of changes including gelatinisation, protein denaturation, colour and flavour changes, oxidation and changes to the nutritional status of the food (Damodaran, 1996; Liu, 1999; Walstra *et al*., 1999).

High pressure or high hydrostatic pressure technology is a non-thermal food processing technology whereby the food is processed by subjecting it to a high hydrostatic pressure without any heat treatment being involved. High pressure may be generated by the addition of free energy, e.g., heating at a constant volume or mechanical volume reduction. It is now technically feasible to reach pressures up to several gigapascals and to keep it constant for a comparably long time in specially designed vessels made from highly alloyed steel (Knorr *et al*., 2006)

General behaviour of biological systems under high pressure is governed by Le Chatelier's principle. This principle predicts that the application of pressure shifts equilibrium towards a state that occupies a smaller volume, and accelerates processes for which the transition state has a smaller volume than the ground state (Balny *et al*., 1997; Huppertz *et al*., 2002; Lullien-Pellerin and Balny, 2002). The volume of atoms are considered to be temperature and pressure independent, it follows that both the thermal expansion and the compressibility are composed of two main terms, cavity volume and hydration volume (Heremans, 2001). Heating has the opposite effect and causes stronger molecular fluctuations and thus an increase in free volume (Heremans, 2001; Knorr *et al*., 2006).

Figure 2.4 defines regions where the protein is native or denatured. The protein is stable in its native state inside the ellipse where ΔG (Gibbs free energy) < 0.

At the equilibrium border between the native and denatures state of the protein, $\Delta G = 0$. At high temperature, pressure stabilizes the protein against temperature denaturation. At room temperature, temperature stabilizes the protein against pressure denaturation (Heremans, 1995; Heremans and Smeller, 1998).

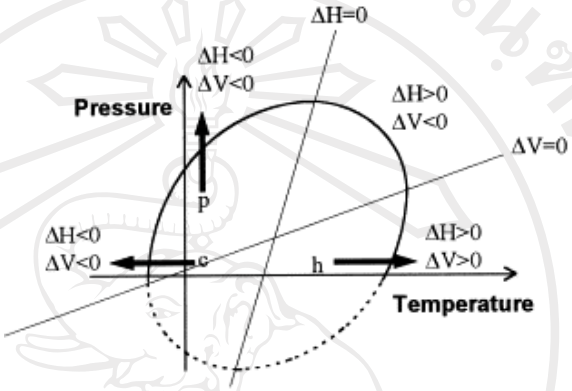

Figure 2.4 Elliptic phase diagram of proteins. The protein is stable in its native state inside the ellipse. The arrows denoted by h, p, c show the three most common denaturation ways, heat, pressure, and cold denaturation respectively (from Heremans and Smeller, 1998).

2.3.2 High temperature protein denaturation

The mechanism of temperature induced protein denaturation involves primarily destrabilisation of the major noncovalent interactions. Hydrogen bonding, electrostatic, and van der Waals interactions are exothermic in nature. They are destabilised at the higher temperatures and stabilised at the lower temperatures. However, since peptide hydrogen bonds in proteins are mostly buried in the interior of the structure, they remain stable over a wide range of temperature conditions. On the other hand, hydrophobic interactions are endothermic. They are stabilised at higher temperatures and destabilized at the lower temperatures. Therefore, as the temperature is increased, the changes in the stability of these two groups of noncovalent interactions oppose each other. However, the stability of hydrophobic interactions cannot increase infinitely with increasing temperature, because above a

certain temperature, gradual breakdown of water structure will eventually destabilize hydrophobic interactions as well. The overall strength of hydrophobic interactions is reported to reach a maximum at about 60-70°C (Damodaran, 1996).

Water greatly facilitates thermal denaturation of the proteins (Ruegg *et al*., 1975). The value of denaturation temperature (Td) decrease rapidly as the water content is increased from 0 to 0.35 g water/g protein. The effect of hydration on thermostability is fundamentally related to the protein dynamics. In the dry state, proteins have a static structure, that is, the mobility of polypeptide segments is restricted. As the water content increased, hydration and partial penetration of water into surface cavities cause swelling of the protein. This swollen state presumably reaches a maximum value at a water content of 0.3-0.4 g/g protein at room temperature. The swelling of the protein increases chain mobility and flexibility, and the protein molecule assumes a more dynamic "fluid" structure. When heated, this dynamic flexible structure provides greater access of water to salt bridges and peptide hydrogen bonds than is possible in the dry state, resulting in a lower Td. (Damodaran, 1996)

Soybean proteins are resistant to heat denaturation. The conglycinin and glycinin exhibit denaturation temperatures of about 75°C and 95°C, respectively (German *et al*., 1982; Yamauchi *et al*., 1991). At temperatures above the denaturation temperature of 11S globulin, the energy supplied is enough to split the disulphide bonds, which significantly increases the α-helix content. The increase in α-helix structure then facilitates the establishment of a good gel matrix (Ker *et al*., 1993).

Utsumi and Kinsella (1985) indicated that in heat treatment, electrostatic interactions and disulfide bonds are all involved in the formation of 11S globulin gels. However, it is mostly hydrogen bonding in 7S globulin gels and hydrogen bonding and hydrophobic interactions in soy isolate gels. Some covalent disulphide bonds are presented in the soy protein gels. However, solubility index did not show an increase in disulphide bond of the samples heated at 90°C for up to 6 hr, whereas the formation of disulphide bond in the heated soy protein gels can be detected using electrophoresis technique (Apichartsrangkoon, 2002).

Thermal treatment is normally used in the various methods to process soybean to inactivate biologically active compounds such as trypsin inhibitors, lipoxygenase, and hemagglutinins, while increasing digestibility of proteins. In addition, thermal treatment helps to diminish the characteristic 'beany' flavour of the raw soy products due to the volatilisation of monocarbonyl compounds, which result from oxidation of the fatty acids by the enzyme lipoxygenase (Estrada-Giron *et al*., 2005).

2.3.3 High pressure and protein denaturation

Pressure induced denaturation of proteins occurs mainly because protein are flexible and compressible. Although amino acid residues are densely packed in the interior of a globular protein, some void spaces invariably exist and this leads to their subsequent compressibity. Pressure induced denaturation of globular proteins is usually accompanied by a reduction in volume which is caused by two factors: the elimination of void spaces as the protein unfolds, and the hydration of the nonpolar amino acid residues that become exposed during the unfolding. The later event results in an overall decrease in volume (Damodaran, 1996).

Of particular interest in food processing are effects of such high pressure on proteins. In their native state, proteins are stabilised by covalent bonds (including disulphide bridges) plus electrostatic interactions (ion pairs, polar groups), hydrogen bonding and hydrophobic interactions (Huppertz *et al*., 2002). High pressure effects on proteins are primarily related to the rupture of non-covalent interaction within protein molecules and their subsequent re-formation of intra-and intermolecular bonds within or between protein molecules (Messens *et al*., 1997). High pressure is able to disrupt electrostatic interactions. Van der Waals forces are presumably favoured by pressure since they intend to maximize the packing density, producing a reduction in volume of the protein (Boonyaratanakornkit *et al*., 2002; Gross and Jaenicke, 1994). For hydrophobic interactions the effect of high pressure depends on the intensity of the pressure: hydrophobic interactions between aliphatic groups are characterised by increases in volume and thus are destabilised at increased pressures (Heremans, 1982). The stacking of aromatic hydrocarbons in water was found to be stabilized by

pressure. Hydrogen bonds, on the other hand, have been found to be virtually insensitive to pressure (Balny *et al*., 1997; Messens *et al*., 1997). Disulphide bonds formation is encouraged quite markedly under pressure (Gomes, 1997). Covalent bonds are almost unaffected by high pressure (Ledward, 1995).

Multimeric proteins, held together by non-covalent bonds, dissociate at relatively low pressures (<150 MPa), thereby disrupting quaternary structures (Huppertz *et al*, 2002). Many quaternary structures are sensitive to pressure such as dissociation which can be followed by aggregation of the subunit or precipitation. The tertiary structure of protein, which is maintained chiefly by hydrophobic and ionic interactions, can be significantly modified at pressures higher than 200 MPa (Messens *et al*., 1997; Hendrickx *et al*., 1998). Changes in secondary structure occur at high pressures and lead to irreversible denaturation, because stabilising hydrogen bonds are enhanced at low pressures and ruptured only at very high pressures (Hendrickx *et al*., 1998).

Overall, the structures of large molecules, such as proteins (including enzymes), may change under the influence of pressure. However, low molecular weight molecules that have little secondary, tertiary and quaternary structure, such as amino acids, vitamins, pigments, flavour and aroma components contributing to the sensory and nutritional quality of food, are largely unaffected (Balci and Wilbey, 1999; Ledward, 1995). These changes depend on protein structure, pressure level, temperature, pH, ionic strength, solvent composition and protein concentration (Boonyaratanakornkit et al., 2002; Lullerin-Pelleria and Balny, 2002).

2.3.4 High pressure studied using soy proteins

At pressure levels greater than 150 MPa proteins aggregation may occur as a result of unfolding because of the higher compressibility and smaller volume of free water as compared with that of protein-water bonds (Dumay *et al*., 1998). High pressure denaturation of β-conglycinin (7S) and glycinin (11S) in soymilk has been reported to occur at 300 MPa and 400 MPa, respectively (Zhang *et al*., 2005). The degree of denaturation of glycinin increasing with increased pressure and resulted in complete unfolding at 500 MPa (Molina *et al*., 2002).

Molina *et al*. (2001) reported that pressure treatment at neutral pH can improve the emulsifying activity of soy proteins. The change of emulsifying activity index of soy proteins after pressure treatment correlated with the surface hydrophobicity. Apichartsrangoon (2003) reported that pressured soy protein of 200 to 800 MPa at 20 and 60°C for 50 min led to little change in the degree of disulphide bonding. Sulphydryl groups as well as hydrophobic regions and amino acid residues of soybean glycinin increased after high pressure processing at ≥ 300 MPa. Some of the ordered structures of α-helix and β-structure in glycinin were destroyed and converted to 'random coils' after processing at 500 MPa for 10 min (Zhang *et al*., 2003).

Dumoulin *et al*. (1998) reported that using pressure treatments of 300-500 MPa at temperatures from -5 to 50°C caused an increase in gelation effectiveness on 17% w/w soy protein system, and this effect increased as the temperature was increased. The solution of soy protein (16.7% w/v) was gelled by the pressurization at 200 MPa and 25°C for 30 min, but this gel lost its shape as soon as it was taken out of the container. Hard gels were only obtained by pressurization at 300 MPa 25°C 30 min or higher (Okamoto *et al*., 1990).

Molina *et al*. (2002) reported that high pressure could only form self-supporting gels for soy protein isolate (SPI), 7S, and 11S at concentrations of about 20% w/v. Lower concentrations did not form self-supporting gels. A combination of heat and pressure on soy proteins led to several differences in the resulting gel structures. When a heat treatment was applied before a high pressure treatment, only 11S globulin could form a self-standing gel. However, when the pressure was applied before the heat treatment, all proteins formed self-standing gels (Molina and Ledward, 2003).

Kajiyama *et al*. (1995) reported that pressurised soymilk processed at 100 MPa for 10 min with added calcium chloride did not form gel at room temperature, whereas treatment at 500 MPa for 10 min produced a gel that was almost twice as strong as an equivalent heat-set tofu. High pressure processing can replace traditional

heat treatment and improve the strength of tofu gel by the creation of a suitable cross-linked network (Zhang *et al*., 2005).

2.4 Dynamic (oscillatory) rheological measurements

Rheology is the science of the deformation and flow of matter (Steffe, 1996; Rao, 1999). It is the study of the manner in which materials respond to the force applied. The force applied is expressed in terms of a stress, which is defined as the force divided by its area of application and usually expressed in Pascal (Pa) or N/m^2 (Steffe, 1996). The deformation produced by the application of such a stress is expressed as the strain, and is the ratio of the size of the deformation of the material when compared to its original size.

The ideal solid or elastic material obeys Hooke's Law, which states that stress (applied force) is proportional to deformation or strain especially when the stress is very small. This differs from the ideal liquid or viscous behaviour material, which obeys Newton's Law of viscosity, which states that the stress is proportional to the rate of deformation.

Foods are classified in a number of different manners, including, solids, gels, homogeneous liquids, suspensions of solids in liquids, and emulsion. Fluid foods are those that do not retain their shape but take the shape of their container. Fluid food contains significant amounts of dissolved high molecular weight compounds and/or suspended solid exhibit non-Newtonian behaviour. Many non-Newtonian foods also exhibit both viscous and elastic properties, that is, they exhibit viscoelastic behaviour (Rao, 1999).

The viscoelastic behaviour of fluids can be determined from dynamic testing where samples are subjected to oscillatory motion when held in various containment systems, usually a cone and plate or a parallel plate apparatus. Typically, a sinusoidal strain is applied to the sample causing some level of stress to be transmitted through the material (Steffe, 1996). Dynamic oscillatory testing techniques are usually non-destructive and have unique advantages for characterizing food systems in their

'undisturbed' states (Bell, 1989). According to Ferry (1980) and Rao (1999), in dynamic rheological measurement, viscoelastic behaviour can be expressed as:

$$G' = (\sigma_0 / \gamma_0) \cos \delta \quad \text{.....(1)}$$

$$G'' = (\sigma_0 / \gamma_0) \sin \delta \quad \text{.....(2)}$$

$$\tan \delta = G''/G' \quad \text{.....(3)}$$

Where G'(Pa) is the storage or elastic modulus, and G"(Pa) is the loss or viscous modulus, tan δ (dimensionless) is the loss tangent, σ_0 (Pa) is the stress, γ_0 (dimensionless) is the strain amplitude, and δ is the phase angle.

The storage modulus (G') expresses the magnitude of the energy that is stored in the material and is recoverable per cycle of deformation. G" is a measure of the energy that is lost as viscous (heat) dissipation per cycle of deformation. Therefore, for a perfectly elastic solid, all the energy is stored; that is, G" is zero and the stress and the strain will be in phase (Figure 2.5). In contrast, for a perfectly liquid material, all the energy is dissipated as heat; that is G' is zero and the stress and the strain will be out of phase by 90° (Figure 2.5).

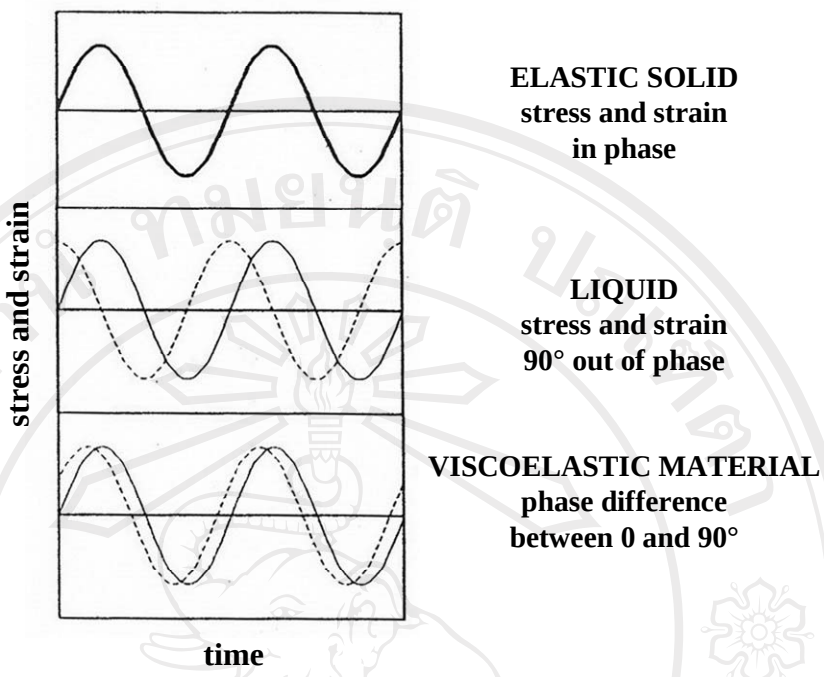

Figure 2.5 Response of different materials to a sinusoidally oscillating stress (from Oakenfull *et al*., 1997).

For a specific food, magnitudes of G' and G" are influenced by frequency, temperature, and strain. For strain value within the linear range of deformation, G' and G" are independent of strain. The loss tangent is the ratio of energy lost to energy stored per cycle of deformation. These viscoelastic functions play important role in the rheology of structured polysaccharides (Rao, 1999).

According to Ferry (1980), materials can be classified into four categories depending on the value of tan δ. (a) For dilute solutions tan δ is very high because both solvent and solute contribute to G"; at low frequencies, tan δ is large because for all uncross-linked polymers and in fact becomes inversely proportional to the frequency. (b) All amorphous polymers, whether cross-linked or not, have values in the neighbourhood of tan δ = 1, ranging from 0.2 to 3. (c) Glassy and crystalline polymers have values near 0.1, and (d) the lightly cross-linked polymer attains extremely small values at low frequencies, of the order of 0.01.

Apichartsrangoon (2002, 2003) reported that heat treated, high pressure treated and untreated soy protein gels exhibited a weak viscoelastic properties. The heat (90°C) treated soy protein gels had higher shear moduli than the unheated ones and the differences increased with increasing time of heating. Overall G' of soy protein displays stronger frequency dependence than G" (Apichartsrangoon, 2002). The storage and loss moduli of soy protein gels increased with increasing pressure (200-800 MPa), however, temperature (60°C) caused rheological modification of the soy protein gels to a greater extent than did increasing pressure (Apichartsrangoon, 2003).

Riblett *et al*. (2001) reported that the rheolograms of gels prepared with β-conglycinin and glycinin shows that G' was greater than G", there was no G'-G" crossover, and G' had a slight frequency dependence from 1 to 10 Hz. Glycinin gels of almost every soybean variety were more elastic than β-conglycinin gels obtained from the same genotype.