Gelation Properties of Protein Mixtures Catalyzed by Transglutaminase Crosslinking

by

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ABSTRACT

Gelation properties of a salt extracted pea (*Pisum sativum*) protein isolate (PPIs) were evaluated with a goal of using this isolate as a meat extender. Microbial transglutaminase (MTG) was used to improve gelation of PPIs, muscle protein isolate (MPI) from chicken breast and the two combined. Gelation properties were evaluated using small amplitude oscillatory rheology and texture analysis. SDS-PAGE and differential scanning calorimetry were used to examine protein structure and thermal properties. Minimum gelation concentration for PPIs was 5%, lower than the 14% obtained for a commercial pea protein isolate (PPIc), possibly because the PPIc was already denatured whereas PPIs was not. Storage modulus (G') and loss modulus (G") increased with protein concentration. Maximum gel stiffness for PPIs occurred at pH 4.0 in 0.3 M NaCl. Higher or lower pH values affected protein charge and the potential for network formation. Higher salt concentrations resulted in increased denaturation temperatures, to a point where the proteins did not denature at the 95ºC temperature used for gel formation. When both heating and cooling rate were increased, gel stiffness decreased, though the cooling rates had a greater impact. Chaotropic salts enhanced gel stiffness, whereas non-chaotropic salts stabilized protein structure and decreased gel formation. Based on effects of guanidine hydrochloride, urea, propylene glycol, β-mercaptoethanol, dithiothreitol and N-ethylmaleimide, hydrophobic and electrostatic interaction and hydrogen bonds were involved in pea protein gel formation but disulfide bond contribution was minimal. Gels formed with MPI at concentrations as low as 0.5% were stiffest at 95ºC, higher than the ~ 65ºC normally used in meat processing. Good gels

were formed at pH 6 with 0.6 to 1.2 M NaCl. Addition of MTG increased gel stiffness and strength for PPIs, MPI, and a combination of the two. SDS-PAGE showed that bands in the 35~100 kDa range became fainter with higher MTG levels but no new bands were found to provide direct evidence of interaction between muscle and pea proteins. Improved gel strength for the MPI/PPI mixture (3:1) containing MTG suggested that some crosslinking occurred. Higher heating temperatures and MTG addition led to the formation of MPI/PPI gel and demonstrated the potential for utilization of pea protein in muscle foods.

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LIST OF ABBREVIATIONS

AACC……American Association of Cereal Chemists International

DSC…….....Differential scanning calorimetry

DTT…… Dithiothreitol

EDTA……Ethylene diamine tetraacetic acid

G-L……..……ε (γ-glutamyl) lysine

GuHCl……Guanidine hydrochloride

HMM……..…… heavy meromyosin

HPLC……...high performance liquid chromatography

LC……...…….Light chain

LMM…… …….light meromyosin

2-ME……β-mercaptoethanol

MHC…….Myosin heavy chain

MLC…….....Myosin light chain

MPI……Myofibrillar protein siolate

MTG……...Microbial transglutaminase

MW……...……Molecular weight

N……..……Newton

NEM……N-ethylmaleimide

PG……..Propylene glycol

SD…….....Standard deviation

PPI……...Pea protein isolate

PPI_S……..Salt-extracted pea protein siolate

PPI_C…….Commercial pea protein siaolte

SPI_C…….Commercial soy protein isolate

SDS-PAGE……..Sodium dodecylsulfate Polyacrylamide gel electrophoresis

TG……..……Transglutaminase

U……...……...…….Unit

Chapter 1: Introduction

Pea (*Pisum sativum*) protein represents a possible substitute for soy protein in meat application; however, the application of pea protein in food products is limited because of its weak functionality as a food ingredient. Gelation is one of the most important properties of plant proteins and affects their application as functional additives in meat products. Consequently, to extend the utilization of pea proteins in muscle foods, it is essential to improve their gelling ability.

As a result of reduced availability and increased prices of meat proteins (Pour-el & Swenson, 1976), demand for plant protein should be high. Compared to plant proteins, animal proteins are expensive due to low conversion rate (about 5 ~ 20% protein in feed is typically converted into edible animal protein) (Bourne, 1982). In contrast, the conversion of plant protein into products providing a meat-like chewy texture can be achieved with losses of only 10 ~ 30% (Bourne, 1982). In addition, demand for low-fat meat-like products in recent years may promote further interest in plant-based protein ingredients. Usually the leanest meat contains about 7% fat compared to < 4% and < 3% in soy protein isolate and pea protein isolate, respectively. Finally, the cholesterol content of meat products could be lowered by replacing a portion of the meat with plant protein (Bourne, 1982).

Gelation of plant proteins is a process involving unfolding and aggregation of proteins. During heating, the proteins unfold, exposing reactive groups, from which intermolecular bonds can be formed with neighbouring protein molecules. When

sufficient bonding occurs, a three-dimensional network is developed, resulting in a gel (Lanier, 2000).

Factors affecting gelation properties of plant proteins include pH, ionic strength, heating and cooling rates, and the molecular forces that are influenced by these factors. Since commercial pea protein isolate is usually extracted by alkaline extraction, acidic precipitation and spray drying, it often undergoes serious denaturation which weakens gel forming ability. A salt-extraction method has been developed for pea protein isolate (PPIs) with minimal denaturation (Arntfield, 2004). Studies which have investigated, at least partially, the gelation of pea proteins tend to use alkali extracted or commercial isolates. Therefore, extensive studies need to be conducted to investigate the impact of these factors on gelation properties of PPIs and to determine the optimum process condition to obtain the strongest PPIs gel.

In addition, when two or more proteins are mixed, there are several ways in which they can interact with each other thereby affecting the properties of the gel formed. Three states (incompatible, semicompatible, or compatible) of the mixed proteins are qualitatively discussed based upon whether two immiscible phases are formed after mixing (Manson & Sperling, 1976).

When pea proteins are incorporated into meat products, the functional properties of pea protein isolates will depend, to a great extent, upon their interaction with the muscle proteins. It has been reported that soy proteins, including the two major globular fractions, β-conglycinin (7S) and glycinin (11S), are resistant to denaturation when used in meat products (Feng & Xiong, 2002; Petruccelli & Añon, 1995a). Under the

normal meat processing conditions (temperature 65-73 ºC, pH 5.5-6.0, and ionic strength 0.1-0.6) none of the major soy globulins demonstrate appreciable structural changes and consequently, interaction with muscle proteins is limited (Ramírez-Suárez & Xiong, 2003a). It was concluded that this lack of interaction reduces the effectiveness of soy proteins as a functional component to improve gel strength and structure in comminuted and emulsified meats (Feng & Xiong, 2002; McCord et al., 1998). Pea proteins also consist of two major components (vicilin, 7S; legumin, 11S) which are very similar to those of soy proteins and have denaturation temperatures above 73 ºC (Shand et al., 2007). Consequently, the application of pea proteins in comminuted and emulsified meats may weaken gel strength and structure due to the lack of interaction between pea proteins and meat proteins.

Microbioal transglutaminase (MTG), a crosslinking enztyme, has been shown to improve and/or induce gelation of a large variety of substrates, including many plant and meat proteins (De Jong & Koppelman, 2002). MTG catalyzes the acyl transfer reaction forming ε-(γ-Glu)-Lys crosslinks (Nonaka et al., 1994; Nielsen, 1995). Partial denaturation of globular proteins prior to MTG treatment was shown to increase the exposure of available reactive groups and enhance the effect of MTG (De Jong & Koppelman, 2002).

The overall objective of this research was to effectively use pea protein isolate as an extender for gels formed by muscle protein. To do this, gelation properties of the PPIs as well as factors affecting gelation characteristics of PPIs including protein concentrations, heating and cooling rates, pH and ionic strength, molecular forces

involved were studied. In addition, conditions (protein concentration, pH, ionic strength and final heating temperature) needed to form MPI gels at temperatures higher than those normally used in meat production were investigated so that the PPIs could be added at temperatures that supported denaturation of these proteins. In addition the effectiveness of a microbial transglutaminase (MTG) as a catalyst to promote interactions between proteins will be evaluated for the PPI/ MPI mixed system and compared to the effects on PPI or MPI alone. The hypothesis is that through the use of higher temperautures and MTG catalyzed crosslinking, gelling ability of MPI/PPIs can be improved thus extending the utilization of PPIs in comminuted muscle foods.

Chapter 2: Literature Review

2.1. Pea production and processing

Pea (*Pisum sativum L.*) is a pulse crop of the family *Leguminacea* that is widely grown in Canada (Pulse Canada, 2010). Canada is the world's leading producer and exporter of peas. Canadian dry pea production was 3.4 million tonnes in 2009-10. Peas are also Canada's largest pulse crop and are mainly grown in the provinces of Saskatchewan, Alberta, and Manitoba. Yellow and green peas are the two major market classes grown in Canada and are exported around the world as ingredients for food processors and canners. Whole and split peas are available as well as pea flour, starch, protein and fibre fractions.

In general, pulses have excellent nutritional benefits. High levels of dietary fibre and complex carbohydrates make them excellent contributors to good health (Pulse Canada, 2010) (Table 2.1). The feed industry uses peas as a source of energy and amino acids that are suitable in diets for all livestock. Peas have been used as feed for long time and are considered a multi-purpose feed ingredient in that they provide both protein and energy. Pea protein concentrate is also used as a protein source for aquaculture diets.

Recent research on the health benefits of peas, beans, lentils and chickpeas and use of whole pulses and pulse flours has increased interest in pulses as an important food ingredient (Pulse Canada, 2010). Growing of pulse crops also has benefits. The rhizobium bacteria living in the root nodules on legumes such as pea, convert the

nitrogen in the air into forms of nitrogen that can be used by the plant. Consequently, the need for commercial nitrogen fertilizer is reduced and agriculture's greenhouse gas emissions from annual crop production are lowered.

Table 2.1 Whole yellow peas nutritional information

Per 100 g dry	Amount
Starch	45.5 g
Protein	23.3 g
Total Fiber	14.7g
Insoluble Fiber	13.1 g
Soluble Fiber	1.57 g
Sucrose	2.6 g
Fat	1.2 g
Calcium	81 mg
Iron	6 mg
Potassium	1230 mg
Vitamin C	0.55 mg
Thiamin	0.51 mg
Riboflavin	0.18 mg
Niacin	1.55 mg
Vitamin B_6	0.05 mg
Folate	33.8 mcg

Pea flour is usually produced by dry milling. Air-classification, another dry processing method, can be used to produce protein concentrates from pea flour. The protein content of commercial pea protein concentrates is around 50-60% (Sathe & Salunkhe, 1981) and when dry processing is used, protein denaturation is minimal. Wet methods are commonly used to prepare pea protein isolates including commercially available pea protein isolate. Proteins are often extracted from pea flour using a range of pH values and subsequently precipitated at the isoelectric point (~ 4.5) (Sathe &

Salunkhe, 1981; Swanson, 1990). Some are then neutralized by alkaline, and finally spray dried. Because of these harsh treatments, commercial pea protein isolates often undergo denaturation, which can greatly decrease their functional properties including gel forming ability. Their final protein content is around 80% (Shand et al., 2008). A method whereby salt extracted protein can be precipitated by dilution in cold water has been applied to fababean (Murray et al., 1985), lentils and peas (Bhatty & Christison, 1984; Ranadheera, 2000) as well as chickpeas (Parades-López et al., 1991). In general, salt-extracted plant proteins were prepared by homogenizing 10-20% (w/v) legume flour suspensions in ~ 0.3 M NaCl for 30 min at room temperature. The soluble plant proteins were then separated by centrifugation (3000-5000×*g*, 15 min) and the supernatant was diluted with cold distilled water (supernatant:water = 1:2-3, v/v). After standing for 6 h at 4 ºC, protein was recovered by centrifugation (3000-5000×*g*, 15 min). The protein was resuspended in distilled water and unwanted salt was removed from the protein concentrate by dialysis. The desalted protein isolate was then freeze dried and a protein isolate with low denaturation was obtained. Isolates containing 87, 91 and 95% protein for lentils, peas and fababean, respectively (Bhatty & Christison, 1984) and lower levels of protein denaturation have been reported (Parades-López et al., 1991).

2.2. Pea protein structure and composition

Dry pea seeds contain approximately 20-27% protein (Wang & Daun, 2004b) of which 65 ~ 70% are the salt extractable globular storage proteins legumin, vicilin and convicilin (Schroeder, 1982). Composition of globular proteins varies among pea

genotypes and the legumin/vicilin ratio has been shown to fluctuate between 0.2 - 1.5 (Casey et al., 1982) with vicilin being the major protein for most cultivars. Subunit composition of the globular proteins within a given cultivar, are also variable as will be discussed in more detail in the successive sections on legumin and vicilin.

2.2.1. Legumin

Legumin is the hexamer (6 subunits) of disulfide linked basic and acidic subunits which fit together as two trigonal antiprisms. Heterogeneity of these legumin polypeptides is a result of the production of legumin precursors from a number of gene families: four/five acidic and five/six basic polypeptides have been identified. It is generally accepted that the molecular weight and isoelectric point (pI) of acidic and basic polypeptides are as follows: around 38 ~ 40 kDa each with pI 4.5 ~ 5.8 for the acidic polypeptides and around 19 ~ 22 kDa each with pI 6.2-8.8 for the basic polypeptides, with different authors reporting different sizes and isoelectric points (Casey, 1979a, b; Krishna et al., 1979; Matta et al., 1981). Variable amino acid compositions have also been reported. Approximately two cysteine and three methionine residues per ~60 kDa subunit were found for the acidic and basic subunits, respectively by Casey & Short (1981), while Croy et al. (1980) reported seven and four.

2.2.2. Vicilin

Pea vicilin is a trimer made of 3 subunits of ~50 kDa (Gatehouse et al., 1981). Smaller polypeptides which have been associated with isolated vicilin are reported to be due to post translational proteolysis (Gatehouse et al., 1983). Fragments of 19 and 30 kDa or 33 and 16 or 12.5 kDa are produced depending on the site of cleavage during

proteolysis (Gatehouse et al., 1982). O'Kane et al. (2004a) separated vicilin into two fractions and named them as vicilin 1º and vicilin 2º and observed that on SDS-PAGE, vicilin 2º contained a third globulin protein, convicilin (~ 70 kDa). They concluded that convicilin was not a separate, third globulin of pea and should be denoted as the α-subunit of the salt extractable pea protein vicilin. This is in contrast to prior work by Gatehouse et al. (1981) who suggested convicilin was a distinct protein (280 kD) made up of 4 ~ 10 kD subunits. Pea vicilin is reported to contain no cysteine and few methionine residues (Thanh & Shibasaki, 1976; Croy et al., 1980).

The small fragments produced by post translational proteolyses contribute to the heterogeneity of vicilin (Casey & Domoney, 1984). Surface charge heterogeneity (around the potential site of cleavage) (Casey & Domoney, 1984) and differential glycosylation (Davey & Dudman, 1979) are other two contributing factors. As the sulfur amino acidic are the nutritionally limiting amino acids, the nutritional value of pea protein can be enhanced by increasing legumin content through plant breeding (Casey & Short, 1981) or by combining with other proteins that have high cysteine and methionine contents.

2.3. Heat-induced gelation of plant proteins

The gel forming ability of proteins has been considered of great importance for the structure of many foods (Bacon et al., 1989), especially comminuted meat products. A process of three consecutive steps are usually used to describe heat-induced gelation of globular proteins: (1) unfolding of the protein by denaturation to expose residues previously buried in the core, (2) aggregate formation by interaction of the exposed

residues, and (3) a continuous network formation by arrangement of the aggregates. A balance of both protein-protein and protein-solvent interactions are essential for heat-induced gel network formation (Arntfield & Murray, 1992). In addition, pH and ionic strength are two main factors which influence these interactions by altering this balance resulting in various types of network (Arntfield & Murray, 1990). Among plant proteins, soy protein was the most widely studied. Renkema (2004) investigated the effect of pH and ionic strength on the storage modulus, fracture strain, and permeability of soy protein isolate gels formed at 0, 0.2 and 0.5 M NaCl at pH 3.8, 5.2 and 7.6. He found that gels with a consistently higher storage modulus and lower fracture strain were formed at pH 3.8 compared with those formed at higher pH values, whereas ionic strength influenced the permeability of the gels (which reflects the pore size of the protein networks) more than pH. He concluded that in addition to strand coarseness, information on the curvature of the strands is needed to relate the rheological properties to the network structures formed under different pH and ionic strength conditions. Because the strands that make up the gel network are composed of protein aggregates, studying how pH and ionic strength affect the consecutive steps of the gelation process (unfolding, aggregation and gel network formation) is especially important to understand the type of gel network formation determined by these factors.

Non-destructive rheological techniques can be used to obtain information on the aggregation process. When an oscillatory strain (deformation) of a fixed dimension is applied to the sample throughout the gelation process, the stress developed over time can be measured. The stress developed is dependent upon the nature of the sample and its intrinsic material properties (Ross-Murphy, 1988). Since the possibility that two

structurally different gels could express the same stress response and cannot be identified by rheological techniques, the employment of microscopic techniques is beneficial for complete understanding of gel networks formation and to confirm the presence of structural features that have been hypothesized based on rheological results.

Without heat-induced unfolding of the globular proteins and exposure of buried residues, protein-protein interactions are unlikely to occur. The kinetics of unfolding and aggregation tend to result in formation of an orderly assembled aggregate when aggregation takes place at a slower rate than unfolding. When aggregation proceeds faster than unfolding, a non-orderly assembled aggregate forms (Arntfield & Murray, 1992). A slow heating rate gives more time for aggregates to interact and assemble themselves in an orderly manner and orderly arrangement of aggregates into a network creates a "fine-stranded network". Gels with transparent characteristic are observed for this type of network (Langton & Hermansson, 1992; Stading & Hermansson, 1991; Doi, 1993; Tani et al., 1993; Tani et al., 1995; Mine, 1996; Matsudomi et al., 1997). In contrast, when the successive processes occur too quickly, larger aggregate clusters are created giving a more random arrangement of aggregates assembles into a network and turbid gels (Langton & Hermansson, 1992; Stading & Hermansson, 1991; Doi, 1993; Tani et al., 1993). The structure of heat-induced gel networks is determined by the intermolecular interactions (Zheng et al., 1993a; Ikeda & Nishinari, 2001). Understanding these interactions is important in studying and modifying the gel texture of related foods.

2.4. Pea protein gelation

As a potential alternative to soy proteins, pea proteins were identified and studies on their functional properties appeared in the literature early in the 60's and 70's. However, little progress has been made on the study of pea protein gelation. In a study focused on heat-induced gelation characteristics, Bora et al. (1994) compared crude and purified pea globulins, legumin and vicilin to a globulin mixture and observed that legumin did not gel, and the amount of legumin in a legumin/vicilin mixture was inversely proportional to the gel hardness. Bacon et al. (1990) compared gelation characteristics of two pea protein isolates (both containing legumin and vicilin) which were prepared through different isolation methods. They found that gels formed in acidic conditions were described as having clarities that made them a suitable replacement for gelatin in vegetarian foods. Bacon et al. (1989) also indicated that at low ionic strength with a pH far from the isoelectric point, pea vicilin could form transparent gels. They explained that under such conditions, electrostatic repulsive forces could be maintained hence the formation of large aggregates could be reduced. O'Kane et al. (2004b) investigated the consequences of compositional heterogeneity on heat-induced gelation of two pea vicilin fractions (vicilin 1º and 2º) and observed that despite having equal opportunity to unfold and expose hydrophobic residues; the minimum gelling concentrations (at pH 7.6) were different (10% w/v for vicilin 1º 14% w/v for vicilin 2º). In addition, the vicilin 1º fraction formed turbid gels while the vicilin 2º fraction formed transparent gels. They concluded that the highly charged N-terminal extension region on the vicilin 2º hindered gelation due to repulsion of the net negative charge. O'Kane et al. (2004c) further studied heat-induced gelation of pea

legumin and observed that gel formation was not affected by changes in the heating rate, and disulfide bonds were not essential within the network strands of these gels. However, disulfide bonds became involved within the legumin network at slower cooling rates. Furthermore, pea legumin gel networks were susceptible to rearrangements that caused the gels to become stronger after reheating/recooling; this was not the case for soy glycinin gel networks. Later O'Kane et al. (2005) investigated gelation behavior of protein isolate extracted from 5 pea cultivars and indicated that the contribution of legumin to the pea protein isolate gels was cultivar specific and related to its disulfide bonding ability rather than the absolute amount of legumin protein content.

Shand et al. (2007) investigated physicochemical and textural properties of heat-induced pea protein isolate gels and obtained the optimal conditions for formation of strong heat-induced gels from commercial pea protein isolate (PPIc) to be a protein concentration of 19.6% (w/w) at pH 7.1 in 2.0% (w/w) NaCl and heating at 93 ºC. They also indicated that commercial soy protein isolate (SPIc) formed stronger and more elastic gels than pea protein under the same conditions. They further investigated the effect of transglutaminase on physicochemical and rheological properties of heat-induced protein gels (Shand et al., 2008) and found that addition of MTG enhanced PPIc gel stiffness and elasticity so that it was similar to SPIc and meat bologna. A positive linear relationship was observed between level of MTG used (0 ~ 0.7%, w/w) and shear stress and shear strain of heat-induced PPIc gels.

Utilization of pea proteins as a potential alternative to soy proteins in food products needs better understanding of their gelation behavior both in the pure states and in food systems. Good understanding of the molecular basis of the gelation mechanisms for pea proteins, especially those mechanisms that determine the structural properties of the gels is essential. In addition, it is also important to determine the effect of environmental factors such as pH, ionic strength, heating and cooling rates on the gelation behavior of pea proteins. Also, the catalyzing effect of MTG on improving pea protein gelation needs to be firther investigated.

2.5. Muscle protein composition

There are different proteins in muscle. These proteins not only constitute the major organic compounds of the muscle tissue, but also are responsible for the structural and biological properties of muscle in living animals (Bandman, 1987). They perform different tasks and have varying properties (Sikorski et al., 1990). Muscle proteins are the major structural and functional components in processed meat system (Smyth et al., 1999) and can be classified into three groups based on solubility characteristics: sarcoplasmic proteins, the metabolic proteins that are soluble in water or dilute salt solutions; myofibrillar proteins, the contractile proteins that are soluble in concentrated salt solutions; and stromal proteins, the connective-tissue proteins that are insoluble in both (Lawrie, 1991).

2.5.1 Sarcoplasmic proteins

The sarcoplasmic proteins refer to the proteins of the sarcoplasm as well as the components of the extracellular fluid and the sarcoplasm. The sarcoplasmic proteins

consist of about 20-35% of the total muscle proteins and are commonly called myogens (Pearson & Young, 1989). Asghar et al. (1985) indicated that sarcoplasmic proteins represent 30-35% of the total muscle proteins or about 5% of the muscle weight. There are around 200 different proteins known to be present in the sarcoplasmic fraction, many of which are glycolytic enzymes responsible for the control of enzymatic reactions in muscle (Kijowski, 2001). Despite their diversity, sarcoplasmic proteins share many common physicochemical properties. Most are of relatively low molecular weight, high isoelectric pH, have globular or rod-shaped structures and low viscosity (Asghar et al., 1985). The sarcoplasmic proteins are extracted by homogenizing the muscle tissue with water or solutions of neutral salts of ionic strength below 0.15.

Myoglobin is probably the most important protein of the sarcoplasm since it is responsible for meat color which is associated with product quality (Kijowski, 2001). Miyaguchi et al. (2000) investigated the thermal and functional properties of porcine sarcoplasmic proteins and found that sarcoplasmic proteins had poor water holding capacity and formed weak and fragile gels.

2.5.2. Stromal proteins

Stromal proteins consist of connective tissue proteins, for instance collagen, elastin, and lipoproteins of cell membrane. They exhibit a fibrous structure, and in the majority of tissues, collagen quantitatively predominates (Kijowski, 2001). Stromal proteins are generally insoluble in dilute solutions of hydrochloric acid or sodium hydroxide (Sikorski et al., 1990) and are the residue after extraction of the sarcoplasmic and myofibrillar proteins. Collagen is composed of three helically twisted polypeptide

chains stabilized by intramolecular and intermolecular bonds and is normally associated with toughness of meats. More covalent bonds are formed inside and between collagen molecules as animals' age, which contribute to toughness of the meats (Asghar et al., 1985; Kijowski, 2001).

2.5.3. Myofibrillar proteins

These proteins can be extracted from the muscle tissue with intermediate or high ionic strength neutral salt buffer usually ranging from 0.30 to 0.70 M. The myofibrillar proteins are related to the water holding capacity and the other functional properties of proteins such as gelation (McCormick, 1994). They comprise about 55-60% of the total muscle protein or 10% of the weight of the skeletal muscle (Asghar et al., 1985). It is well known that myofibrillar proteins are mainly responsible for the textural properties of processed meat products (Asghar et al., 1985; Yasui et al., 1980). The adequate extraction of myofibrillar proteins is particularly important for maintaining gel forming ability in meat products (Li-Chan et al., 1987). Of the myofibrillar proteins, myosin and actin are two major proteins responsible for muscle contraction in the living animal, as well as many functional characteristics in processed meat products. Contractile proteins which are different in size and location in the muscle are listed in Table 2.2 (Ashie & Simpson, 1997).

Table 2.2 Contractile proteins in food myosystems

Proteins	Relative content (%)	Size (kDa)	Location
Myosin	50 ~ 60	470	Thick filaments
Actin	15 ~ 30	43 ~ 48	Thick filaments
Tropomyosin	5	65 ~ 70	Thick filaments
Troponins	5		Thin filaments
Troponin-C		17 ~ 18	
Troponin-I		20 ~ 24	
Troponin-T		37 ~ 40	
C-protein	-	140	Thick filaments
α-Actin	-	180 ~ 206	Z-disc
Z-nin	-	300 ~ 400	Z-disc
Connective/Titin	5	700 ~ 1000	Gap filaments
Nebulin	5	~ 600	N_2-line

Adapted from © Ashie & Simpson (1997) with permission from Ashie & Simpson on Jan. 11, 2011.

2.5.3.1 Myosin

The thick myofilaments of the sarcomeres are primarily composed of myosin. Some researchers reported myosin consists of 43 ~ 45% of the myofibrillar proteins in the muscle of mammals, birds, and fish (Yates & Greaser, 1983; Maruyama, 1985), while others indicated that myosin makes up 50 ~ 58% of the myofibrillar fraction (Sikorski et al., 1990). This difference is probably caused by different animal origin and extraction methods. Myosin is a large fibrous molecule and its native molecular weight is about 500 kDa. Myosin consists of six polypeptide subunits; two large heavy chains (myosin heavy chain, MHC) and four light chains (myosin light chain, MLC) arranged

into an asymmetrical molecule with two pear-shaped globular heads attached to long α-helical rod-like tail (Xiong, 1997) (Fig. 2.1).

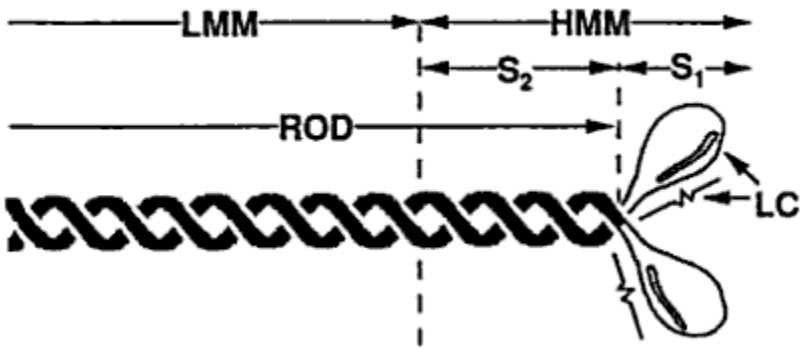

Fig. 2.1 Myosin molecule model. Reprinted from Hettiarachchy & Ziegler (1994) *Protein Functionality in Food System* with permission.

Myosin exhibits three important biological properties in living muscle. First, myosin molecules can assemble themselves and build filaments. Second, through the reaction of ATPase at the catalytic site of myosin head energy can be provided for muscle contraction. Third, myosin forms natural complexes with actin, the main component of the thin filament. This interaction is important for the generation of the force that moves the thick and thin filaments past each other (Stryer, 1995).

To extract myosin, higher than 0.15 M salt (NaCl or KCl) buffer is usually used. $MgCl_2$ and ATP or pyrophosphate can be added into the buffer to prevent simultaneous extraction of actin. Because of oxidation of thiol groups, myosin molecules tend to aggregate. To prevent its aggregation, ethylenediaminetetraacetic acid (EDTA) and

mercaptoethanol can be added (Kijowski, 2001). The myosin molecule dissociates into subunits of high and low molecular weight under the effect of sodium dodecylsulfate (SDS) that can be separated by electrophoresis.

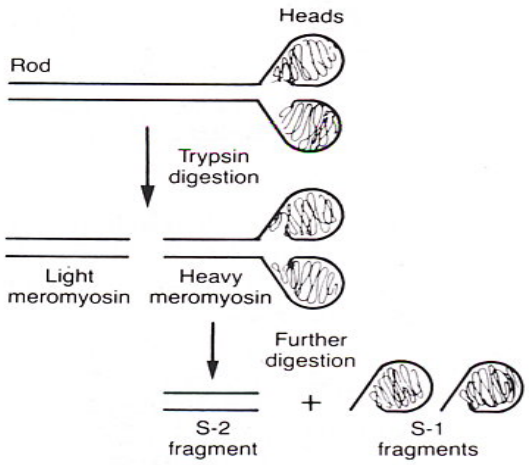

Figure 2.2 Schematic of the generation of LMM, HMM, S-1, and S-2 fragments. Reproduced from ©Bechtel (1986) with permission from Bechtel on Nov. 9, 2010.

Myosin molecules are often broken down to smaller fragments to facilitate studies since myosin is a large complex protein. The enzymes trypsin, papain, or chymotrypsin are widely used to cleave myosin (Lowey et al., 1969; Weeds & Pope, 1977). Trypsin or chymotrypsin can split myosin into two functional fragments: a slow sedimenting component called light meromyosin (LMM, 150 kDa) and a rapid sedimenting component called heavy meromyosin (HMM, 350 kDa). Trypsin can further digest HMM to a head part called S-1 (115 kDa) and a neck part called S-2 (60 kDa), respectively (Young et al., 1965; Bechtel, 1986) (Figure 2.2). The S-1 subfragment contains an ATPase region, a region to bind actin, and two regions to bind light chains (Bechtel, 1986) and it has the ability to bind actin thin filaments and

produces muscle contraction (Stryer, 1995). The light chains bind to the α-helical regions of the heavy chain and the tail portion of the heavy chain molecule is responsible for its association into thick filaments (Foegeding et al., 1996). The thick filaments are composed mainly of hundreds of myosin molecules. Muscle myosin contains two heavy chains (MHC) (*M*r~220 kDa) and four light chains (MLC) (*M*r~20 kDa).

The long tail of the myosin molecule consists of two polypeptides in a coiled α-helix-terminating in two globular heads at one end (McCormick, 1994). Consequently, due to its excellent binding capacity, the S-1 subfragment may play a key role in the functionality of myosin in processed muscle foods (Borejdo, 1983; Borejdo & Assulin, 1980).

Abundant glutamic acid and aspartic acid residues and a fair amount of the basic residues histidine, lysine, and arginine are found in myosin (Harrington, 1979). Under normal meat processing conditions where the pH value is around 6, the myosin molecule will be negatively charged and has the ability to bind water because the isoelectric point of myosin is around 5.3 (Harrington, 1979). By increasing the effective net negative charge, breaking ionic bonds, and causing molecular swelling and water uptake, salt will further enhance the water-binding ability of myosin (Acton et al., 1983). The functionality of myosin in processed meat products will be reviewed in the protein gelation section.

2.5.3.2 Actin

Actin accounts for 15~22% of the myofibrillar protein and is the main component of the thin myofilaments (Yates & Greaser, 1983; Sikorski et al., 1990). Each actin molecule normally appears as a globular protein, called G-actin with a molecular weight of about 40 kDa. Another type of actin is F-actin, which is compsed of actin molecules that have been polymerized via covalent interactions to produce helical filamentous molecules. A double helix, called the thin filament or I-band, which associates with tropomyosin and troponin, can be formed by two F-actins wrapping around each other (McCormick, 1994).

2.5.3.3 Actomyosin

A complex, called actomysin can be formed when actin and myosin are mixed *in vitro* and this complex can be dissociated by the addition of ATP. Since ATP is exhausted by postmortem metabolism, actomyosin is the main state of actin and myosin in postmortem muscle (Ochiai & Chow, 2000).

2.5.3.4 Tropomyosin

Tropomyosin consists of two polypeptide chains, each with a molecular weight range of 34-36 kDa, which associate to form a coiled helix, or a rod-like molecule. The tropomyosin molecule is around 385 Å long and associates in a head-to-tail mode to form a filament that follows and associates with the coil of the F-actin filament (McCormick, 1994). A tropomyosin molecule interacts with 7 molecules of G-actins (Foegeding et al., 1996).

2.5.3.5 Troponin

Troponin is an asymmetrical protein and consists of three subunits. The subunit troponin T, which has a molecular weight of 37 kDa, is also bound to troponin subunits C and I, and links the troponin molecule to the tropomyosin molecule in the I-band. Troponin C has a molecular weight of 18 kDa, binds Ca^{2+} and confers Ca^{2+} sensitivity to the troponin-tropomyosin-actin complex. Troponin I is the inhibitory subunit, has a molecular weight of 23 kDa, binds tightly to troponin and actin and binds only slightly to tropomyosin or troponin T (McCormick, 1994).

2.6. Gelation of muscle protein

Gelation is important for meat product texture. Excellent gels can be formed by myosin alone. Actin has a synergistic or antagonistic effect on myosin gelation depending upon the ratio of myosin/actin in the gelling system (Grabowska & Sikorski, 1976; Matsumoto, 1980). The characteristics of protein gels are different and dependent on factors such as protein concentration and degree of denaturation caused by temperature, pH, and ionic strength (Totosaus et al., 2002). Protein gelation can be achieved in many ways, among which, heat-induced gelation is the most common method. A three-dimensional gel network which provides both structural and functional properties to meat products can be formed by meat proteins upon heating (Acton et al., 1983). Both intramolecular (conformational) and intermolecular changes in proteins are involved in thermally induced gelation and the mechanism of gel formation may differ among proteins probably due to the types of molecular interactions that stabilize the gels. These interactions include protein-protein, protein-water, and protein-fat

interaction (Acton & Dick, 1989). Multiple hydrogen bonds (Eldridge & Ferry, 1954), disulfide linkages (Huggins et al., 1951), peptide bonds (Bello, 1965) as well as electrostatic and hydrophobic interactions may be involved in these associations (Wolf & Tamura, 1969).

Of the three major protein groups in muscle (Smith, 1988), myofibrillar proteins are the most important to the development of the gel structures in heat-processed products. In restructured, formed and comminuted meat products, gelation of myofibrillar proteins is also responsible for texture, juiciness, and stabilization of fat emulsions in processed meat products (Xiong, 1997). Denaturation and aggregation are the two steps of muscle protein gelation (Ziegler & Aton, 1984).

2.6.1. Protein denaturation

Many factors can induce protein denaturation and heating is a major one which is usually used to induce denaturation and gelation of muscle proteins. As a result of various treatment conditions the native protein structure may undergo conformational changes. For example, meat proteins may denature due to exposure to heat, and changes in pH and ionic strength during food processing. Anglemier & Montgomery (1976) defined denaturation as "continuous process of native protein structural changes involving the secondary, tertiary, or quaternary structure during which alteration of hydrogen bonding, hydrophobic interactions, ionic linkages and oxidation-reduction or interchange reactions of covalent disulfide bonds occur without alteration of the amino acid sequence". Changes associated with the heat-induced denaturation of actomyosin are shown in Table 2.3.

Table 2.3 Conformational changes occurring during the thermal denaturation of natural actomyosin

Temperature	Protein (s)	Description of events
30~35	Native tropomyosin	Thermal dissociation from the F-actin backbone
38	F-actin	Super helix dissociates into single chain
40~45	Myosin	Dissociated into light and heavy chains
	Head	Possibly some conformational change
	Hinge	Helix to random coil transformation
45~50	Actin, myosin	Actin-myosin complex dissociates
50~55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

From Ziegler & Acton (1984) with permission.

The relatively weak forces that hold proteins in their folded and helical tertiary and secondary configurations can be broken when energy is conveyed to the protein molecules upon heating. The protein molecules thus unfold and the internally directed hydrophobic regions are exposed to the outside of the molecules. Interactions between the exposed hydrophobic sites become inevitable when many hydrophobic sites are exposed; this induces protein aggregation (gelation) (Nakai, 1983).

Several conformational transitions in structure occur during thermal denaturation of a protein (Lesiów & Xiong, 2001a). To identify points where conformational changes in the protein occur upon the absorption of thermal energy, transition temperatures (designated as T_m) for different muscle proteins have been used. T_m of myosin, the major muscle protein has been extensively studied (Dudziak &

Foegeding, 1988; Liu & Foegeding, 1996; Smyth et al., 1996). During heating, the myosin molecule undergoes two major transitions: the first one is denaturation of myosin heads, and the second one is disassociation of myosin rods (Burke et al., 1973; Samejima et al., 1976). Due to different experimental conditions, for instance, muscle type, ionic strength, and pH, discrepancies among transition temperatures have been reported in the literature (Lesiów & Xiong, 2001b). The second transition (~ 55 °C) is probably the more critical, because gels do not achieve appreciable strength until this temperature is reached (Ziegler & Acton, 1984).

2.6.2. Aggregation

The denatured protein molecules which have unfolded and re-oriented themselves, interact at specific points and finally form an ordered three-dimensional network structure during heating (Foegeding, 1988). Myosin is a predominant protein responsible for gelation of muscle. Samejima et al. (1981) suggested that the heat-induced gelation of myosin comprises two reactions based on the observations of heat-induced gelation characteristics of myosin and its proteolytic subfragments: (1) aggregation of the globular head portion of the molecule which is associated with the oxidation of SH groups; and (2) network formation following the thermal unfolding of the helical tail portion. Also, formation of "super-junctions" which provide extra cross-linking within the gel network formed by association of the head portions was proposed by these authors. Based on the above information, we present a diagram of gel network formation by myosin (Fig. 2.3). Myosin heads become joined by disulfide bonds and may lose shape during denaturation. The helical myosin rod forms β-sheets and random coils during acid and heat denaturation. The light meromyosin rod may detach from the

head and be involved in network formation. Actin contributes to viscosity but does not appear to be involved in network structure.

It appears that while gelation of the heat-induced myosin occurs in two phases, the mechanism of gelation may differ among protein sources. Changes in protein conformation (denaturation) are the major events at temperatures below 55 °C and at approximately 55 °C when the myosin rods start to aggregate, gelation begins. To form an ordered gel, the aggregation rate needs to remain lower than the denaturation step (Totosaus et al., 2002).

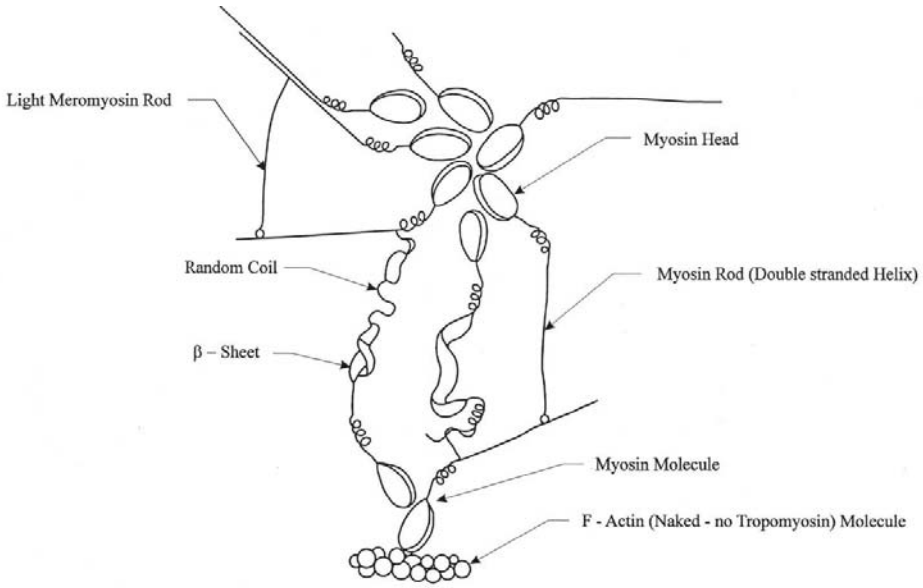

Fig. 2.3 Diagramatic representation of gel network formation by myosin.

2.7. Transglutaminase catalyzed crosslinking

One of the most extensively used agents to form protein gels, especially in meat systems, is the enzyme transglutaminase (TG). It is a protein γ-glutamyltransferase, EC 2.3.2.13, capable of catalyzing acyl transfer reactions and introducing covalent cross-

links between proteins (Nio et al., 1986; Nonaka et al., 1989) (Fig. 2.4). TG has been extracted from both animal and microbial sources. Calcium-dependent TG, extracted from blood plasma and guinea-pig liver, has been investigated for use in the food industry (Kurth & Rogers 1984; Kim et al., 1993). However, poor availability, complicated separation and purification procedures, as well as the requirement for calcium have made the application of mammalian TG in food processing on an industrial scale very difficult. Microbial transglutaminase (MTG), on the other hand, is easily obtained by microbial fermentation and it can be produced in large enough quantities for commercial use (De Jong & Koppelman 2002). In addition, MTG does not require calcium for activation (Sakamoto et al., 1994), which is of great advantage as many food proteins precipitate in the presence of Ca^{2+}, thus rendering them less sensitive to the enzymatic reaction.

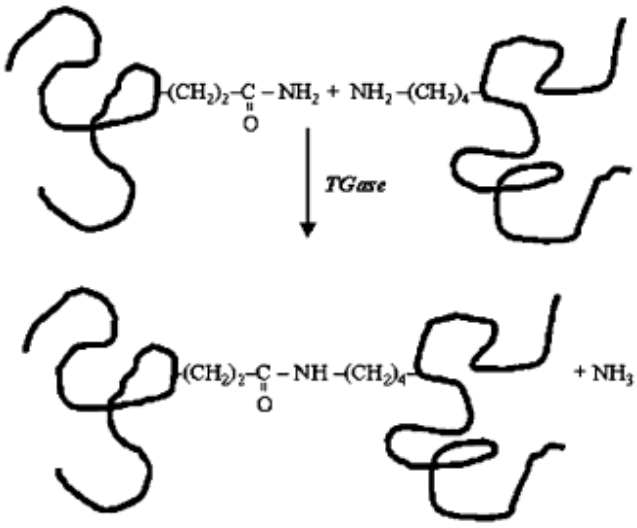

Fig. 2.4 Cross-link by transglutaminase formation of ε-(γ-glutamine)-lysine between lysine and glutamine residues.

MTG has been used in restructured and extended meat products (Kuraishi et al., 1997; Ramírez-Suárez & Xiong, 2002, 2003a), as well as for the gelation of fish proteins and surimi products (Sakamoto et al., 1994, 1995; Lee et al., 1997; Jiang et al., 1998, 2000; Tsujioka et al., 2005) and for the gelation of shrimp (Tammatinna et al., 2007). Dondero et al. (2006) indicated that MTG addition of gel which occurred at 60 ºC after 2 h with 0.5% (w/w) MTG significantly increased beef protein gel breaking strength with a gel strength 88% higher than the control. Although some studies have reported that excessive TG can lead to undesirable outcomes such as reduced gel strength of whey proteins (Truong et al., 2004), or reduced cohesiveness of soy gels (Nonaka et al., 1994), no adverse effects have been reported with excessive use of MTG on meat proteins. In a study by Ramírez-Suárez & Xiong (2002), all treatments were incubated with 0.1% (w/v) MTG at 5 °C and analyzed after reacting for as long as 15 h. They concluded that MTG causes cross-linking of myosin and produces a gel network with improved elasticity by modifying both intra- and intermolecular interactions. In another study, Ramírez-Suárez & Xiong (2003a) observed that myofibrillar protein isolate (MPI) could cross-link with soy protein isolate (SPI) in the presence of MTG (0.1%, w/v) incubated at 5 °C for up to 240 min. The enzyme treatment greatly enhanced the elasticity of gels prepared from MPI/SPI mixtures 3:1, 1:1, and 1:3 compared with untreated samples.

Ahhmed et al. (2009a) investigated the factors that caused differences in improvement of gel strength in chicken and beef myofibrillar proteins after addition of

MTG. It was observed that the elasticity was weaker in the chicken samples than the beef samples. Their results suggested that due to physiological muscles and fiber type, biological substrate, and biochemical inhibitors and amino acids variables, MTG exhibited different ability to access chicken and beef myofibrils. In further research by Ahhmed et al. (2009b), the difference in interaction between chicken and beef actomyosin, myosin B with MTG was investigated. It was found that MTG improved the gel strength of myosin B in both species and confirmed that the gel strength of beef was significantly greater than chicken. They suggested that the reactivity of MTG was dependent upon the residual amino acids present on the surface of myosin B in meat. They also indicated that some protein components joined by the disulfide bonds of cysteine in chicken peptides with long reiterated methylene groups were inhibitory and reduced MTG activity. They concluded that in chicken and beef, the optimal cross-linking levels induced by MTG are due to differences in myosin B.

Herrero et al. (2008) investigated the effect upon secondary structure of adding MTG to meat systems. It was found that its addition produced a significant increase in hardness, springiness, and cohesiveness in the meat systems. Following addition of MTG, a significant decrease in α-helix content, together with a significant increase in β-sheets and turns, were found by Raman spectroscopy. It was also found that there was a significant correlation between these secondary structural changes and the textural properties hardness, adhesiveness, springiness, and cohesiveness of meat systems.

2.8. Interaction of meat/nonmeat protein

Protein additives such as egg white, soybeans, and whey can enhance gel characteristics of meat systems, with egg white showing the most benefit by producing the hardest gel (Chang-Lee et al., 1990); however, there usually is a lack of interaction between nonmeat proteins and muscle proteins in processed meat products. As a result, these nonmeat proteins may not participate in structure development and can negatively affect texture by interfering with the gelation of the myofibrillar proteins (Foegeding & Lanier, 1989).

Muguruma et al. (2003) investigated shear force of chicken sausages following the addition of soy protein isolate, casein, whey protein isolate, and their mixtures in the presence of 0.05 or 0.2% sodium tripolyphosphate (STPP). The texture of chicken sausages was improved by the addition of these biopolymers even in the presence of 0.05% STPP. They suggested that with the addition of biopolymers, the formation of network structures induced by MTG improved the hardness of chicken sausage gels and enabled a reduction in phosphate content without a loss in texture. Chin et al. (2009) investigated the impact of soy protein isolate (SPI) substitution for sodium caseinate (SC) on the gelation properties of cold-set (4 °C) and heat-induced gels of pork myofibrillar protein (MP) in the presence of MTG. With increased MTG incubation time, the strength of cold-set MP–SC gels formed in 0.45 M NaCl, 50 mM phosphate buffer, pH 6.25, increased, but the gel strength of those with 66% or more SPI substituted for SC had strength reduced by more than 26%. Saio & Watanabe (1978) also reported that the interaction between soy 11S protein and muscle protein resulted

in improved MPI gelling ability. However, it has also been reported that the interaction between 11S protein and myosin only occurs when the 11S components are partially or fully denatured (Peng et al., 1982a, b).

Burgarella et al. (1985) reported that egg white and whey protein substitutions interfered with fish protein gelation in surimi. The energy levels required to penetrate the fish + egg white and fish + whey gels were lower than those required for individual protein gels. Hongsprabhas & Barbut (1999), on the other hand, indicated that using unheated whey protein isolate (WPI) at a substitution level of 2% of the meat proteins did not cause any detrimental effect on meat gelation properties. In fact, they showed that substitution with preheated WPI was beneficial and resulted in increased WHC, reduced cook loss, and increased gel strength of the raw and cooked products, particularly at low salt levels. Ramírez-Suárez & Xiong (2002) found that combining WPI and MPI weakened gels heated between 65 °C and 75 °C, but when the temperature was above 77 °C, a stronger gel was formed from the mixture. They felt the denaturation of β-lactoglobulin at this temperature may have been responsible for this improvement. Inclusion of MTG in this system did not promote cross-linking between the whey and muscle proteins, but did appear to promote cross-linking within the myofibrillar protein gel network, resulting in an overall improvement in gel properties.

In general, a lack of interaction between meat and nonmeat proteins results in reduced gel strength of protein mixtures; however, higher heating temperature or preheating can induce denaturation of soy 11S protein and WPI β-lactoglobulin, which

enable interactions between meat and nonmeat proteins, thus increase the gel strength of protein mixtures. Although a number of studies have focused on the effects of plant proteins especially soy proteins on gelation properties of comminuted meat products, limited studies (Verma et al., 1984; Su et al., 2000; Serdaroglu et al., 2005; Pietrasik & Janz, 2010; Sanjeewa et al., 2010) were conducted to incorporate pea protein into comminuted meat products.

Verma et al. (1984) used chickpea flour in 'English' type fresh skinless sausages (mutton, pork or beef) and observed that the acceptability of mutton sausages containing chickpea flour was not affected at levels of substitution up to 40% on a protein to protein basis. In contrast, at substitution levels above 30%, pork and beef sausages were significantly less acceptable. Inclusion of chickpea flour resulted in increased cooking losses and softer textures in all the sausages.

Su et al. (2000) investigated functional properties and microstructure of frankfurters containing 15% pre-emulsified fat (PEF) stabilized with 2% pea protein isolate, soy protein isolate, or sodium caseinate and observed that with the exception of frankfurters with pea protein, all the others made with PEF had greater thermal stability than all-meat frankfurters. Frankfurters containing pea protein had weaker shear force than those with soy protein or sodium caseinate. Based on and examination of the microstructure, many fat globules were entrapped in soy protein or sodium caseinate networks, which stabilized the meat emulsions and contributed to a firmer texture. They concluded that pea protein had a weakening effect on the texture of the frankfurters with PEF.

Serdaroglu et al. (2005) used blackeye bean flour (BBF), chickpea flour (CF), lentil flour (LF) and rusk (R) in meatballs as an extender at level of 10% and observed that meatballs extended with BBF and CF had higher water holding capacity than other treatment groups. All meatballs incorporating legume flours were tougher (lower penetration values) than the R treatment. The authors concluded that these legume flours slightly increased toughness of meatballs and suggested that legume flours can be successfully used in meatball formulations as extenders. However, there were no all-meat meatballs or meatballs made using soy protein as controls, thus making it difficult to compare these results to other studies.

Pietrasik & Janz (2010) investigated the influence of pea flour, starch, and fiber on functionality, quality, and acceptability of low fat bologna (LFB) and observed that all pea ingredients lowered cooking and purge losses, and increased water holding capacity compared to all-meat LFB. However, the hardness, springiness, chewiness, and fracturability of LFB with pea flour were the lowest compared to the other treatments. Consumer acceptance of LFB extended with pea starch and fiber fractions was equivalent to high fat bologna (HFB) whereas pea flour resulted in the lowest consumer acceptability.

Sanjeewa et al. (2010) investigated the suitability of chickpea (one Kabuli and Desi variety) grown in Western-Canada in a low-fat (fat <5%) pork bologna using pea and wheat flour for comparison. Their results demonstrated that incorporation of chickpea or pea flour into a low-fat pork bologna at levels 2.5% and 5% increased the product's cook yield, the instrumental hardness, springiness, and chewiness and sensory

firmness, and decreased water releasing properties. However, chickpea flours were shown to be superior to pea flours in this application. Their study demonstrated, however that both chickpea and pea flour have good potential as extenders in low-fat emulsion-type meat systems.

In general, none of the above studies investigated the interactions between pea protein and myofibrillar protein based on dynamic rheological measurement and SDS-PAGE analysis. Therefore, this is what to be focused on in this study.

Chapter 3: Gelation properties of salt extracted pea protein induced by heat treatment (Sun, X. D. and Arntfield S. D. 2010. *Food Research International*, 43, 509-515)

3.1. Abstract

Gelation is one of the most important properties of plant proteins. In this paper, a low denaturation salt extraction method was used to extract pea (*Pisum sativum*) protein isolate from commercial pea flour. The gelation properties of this isolate were examined and compared to commercial products. The pea protein isolate (PPI) followed the three-step process of gelation that is generally accepted for heat-induced gelation of globular proteins. The minimum gelation concentration of salt extracted pea protein isolate (PPIs) was 5.5% while that of commercial pea protein isolate (PPIc) was 14.5%. The gelling point was in the range of 82~86ºC for 14.5% PPIs, 0.3M NaCl at natural pH (5.65). With increasing heating rate, the gelling point tended to increase; higher heating and cooling rate resulted in decreased final G' (storage modulus) and G" (loss modulus) values, indicative of decreased gel stiffness. Higher protein concentration resulted in higher G' and G" values and it was found that there were power law relationship between protein concentration and G', G". Tan delta (G"/G') values decreased with increasing protein concentration and at concentration of 5.5% and up, tan delta remained constant which means the critical concentration for gel formation is 5.5%. When compared to commercial pea protein isoalte (PPIc) and soy protein isolate (SPIc), the values of G' and G" for PPIs were greater than those of PPIc, and tan delta of PPIs was smaller, indicative of a stiffer gel network. DSC data showed that PPIc underwent denaturation whereas

PPIs had not (ΔH =15.81 J/g protein). Although rheometer data showed that the final G' value of SPIc was lower than that of PPIs, the gel prepared with SPIc was visually more uniform than that of PPIs. The rheological data obtained with small amplitude oscillatory testing was not consistent with the actual observation. Overall, the low denaturation degree of the PPIs resulted in a stiffer gel than that of PPIc making the PPIs a more attractive food ingredient.

3.2. Introduction

Dry peas can be processed into fractions including pea flour, pea protein isolate (PPI), pea starch, and pea fiber. The application of pea protein in food products, however, is limited because of its weak functionality as a food ingredient. In the literature, we rarely find reports on gelation properties of PPI. If used as a substitute for meat proteins or as a nutritious and functional additive, pea protein can play an important role similar to what is done with soy protein. However, pea protein isolate forms a weaker and less elastic gel than soy protein isolate when processed using the same conditions (Shand et al., 2007).

Globular proteins from various sources play important roles in many foodstuffs, both because of their nutritional value and their contribution to food texture (van Kleef, 1986). These textural contributions come from the network structures created by the proteins. Since gelation is important functional property of the globular proteins used to modify food texture (Ikeda & Nishinari, 2001), it is essential to understand which factors determine the gel network and how they are affected by processing parameters. Such an understanding would enable better control of food texture.

For globular proteins, protein-protein interactions normally occur following denaturation, often induced by heating. The pH, presence of ionic species, heating temperature, and heating rate are factors that affect gel network formation by globular proteins (Matsumura & Mori, 1996). In fact, these are processing parameters that can be manipulated for gel formation.

Small strain oscillatory (dynamic) testing is useful in evaluating gelation properties and gel stiffness, because this method is extremely sensitive to changes in physical structure and chemical composition of the sample (Ross-Murphy, 1984). The method is suitable for measurement of subtle changes associated with gel forming phenomenon (Hamann, 1987). High G' (storage modulus) values demonstrate stronger intermolecular network and increased interactions between protein-protein and protein-polysaccharide molecules, while low tan δ values indicate a more elastic network (Uruakpa & Arntfield, 2006).

The gelling point determination from oscillatory measurements has been tested by several different methods, including the crossover of the storage (or elastic) modulus G' and the loss (or viscous) modulus G" (Clark, 1991; Friedrich & Heymann, 1988; Ikeda & Nishinari, 2001; Winter, 1987), linear extrapolation of the rapidly rising storage modulus G' to the intercept with the time axis (Hsieh et al., 1993; Steventon et al., 1991). After the gel point, protein aggregates are bound together into a continuous molecular structure, as described by Hsieh & Regenstein (1992). The formation of these aggregates is responsible for the changes monitored.

Both globulins and albumins in pea protein isolates contribute to gel formation (Shand et al., 2008). Physicochemical and textural properties of heat-induced pea protein isolate gels were studied by Shand et al. (2007). Studies on the gelation properties of pea mixed globulins, vicilin and legumin have been reported by Bora et al. (1994), O'Kane et al. (2004a, 2004b) and O'Kane et al. (2005) It was found by Bora et al. (1994) that pea globulin underwent heat induced gelation while legumin did not gel under the same condition whereas O'Kane et al. (2004a, 2004b, 2005) indicated that both pea vicilin and legumin could form gels. This is probably caused by different pea cultivars since O'Kane et al. (2005) indicated that the contribution of legumin to the pea protein gels was cultivar specific. Although studies on gelation properties had been done on the basis of pea protein isolate, purified pea vicilin and legumin, the extraction method of pea protein isolate was isoelectric precipitation, none of these studies investigated gelation properties of pea protein isolate that had been prepared using a salt extraction method.

The objectives of this study were to investigate the gelation properties of salt extracted pea protein induced by heat treatment and determine the impact of heating and cooling rate and protein concentration on these properties. The gelation properties of commercial pea protein isolate and soy protein isolate were also determined for comparison.

3.3. Materials and methods

3.3.1. Commercial pea flour, PPIc, SPIc and PPIs extraction procedure

Commercial pea flour and pea protein isolate were kindly donated by Nutri-Pea Ltd (Portage la Prairie, MB, Canada). The Century Flour was made from Canadian

yellow pea by the wet milling method, protein content was greater than 25%, carbohydrate content less than 75%, starch content around 50%. The commercial pea protein isolate (PPIc) —Propulse Pea Protein contained 82% protein, carbohydrate less than 12% and starch less than 0.7% as reported by the supplier. Commercial soy protein isolate (SPIc) (PRO-FAM® 974 soy protein) was obtained from Archer Daniels Midland Company (ADM) (Decatur, IL, U.S.A.) and protein content was 90%, total carbohydrate content less than 1%, as reported by the supplier. The pea protein isolate prepared by salt extraction method (PPIs) after freeze drying (Genesis SQ Freeze Dryer, Gardiner, NY, U.S.A.) contained 81.9% of protein as determined by Kjeldahl method using an N to protein conversion factor of 5.7 (AACC, 1982). Salt extracted pea protein procedure is shown in Fig. 3.1.

3.3.2. Minimum gelling concentration

Minimum gelling concentration was determined by a procedure adapted from the method of O'Kane et al. (2005) with a slight modification. Protein solutions (5 mL) were made at concentrations of 4-18% (w/v) for the salt extracted pea protein and 8-20% for the commercial pea protein isolate. All samples were dissolved in 0.3 M NaCl. All samples were heated (in sealed tubes to avoid evaporation) at 95 °C in a water bath for 10 min. Samples were cooled to room temperature for 1 h, and then stored at 4 °C overnight. The next day the tubes were inverted and the lowest concentration sample that did not flow was considered to be the minimum gelling concentration.

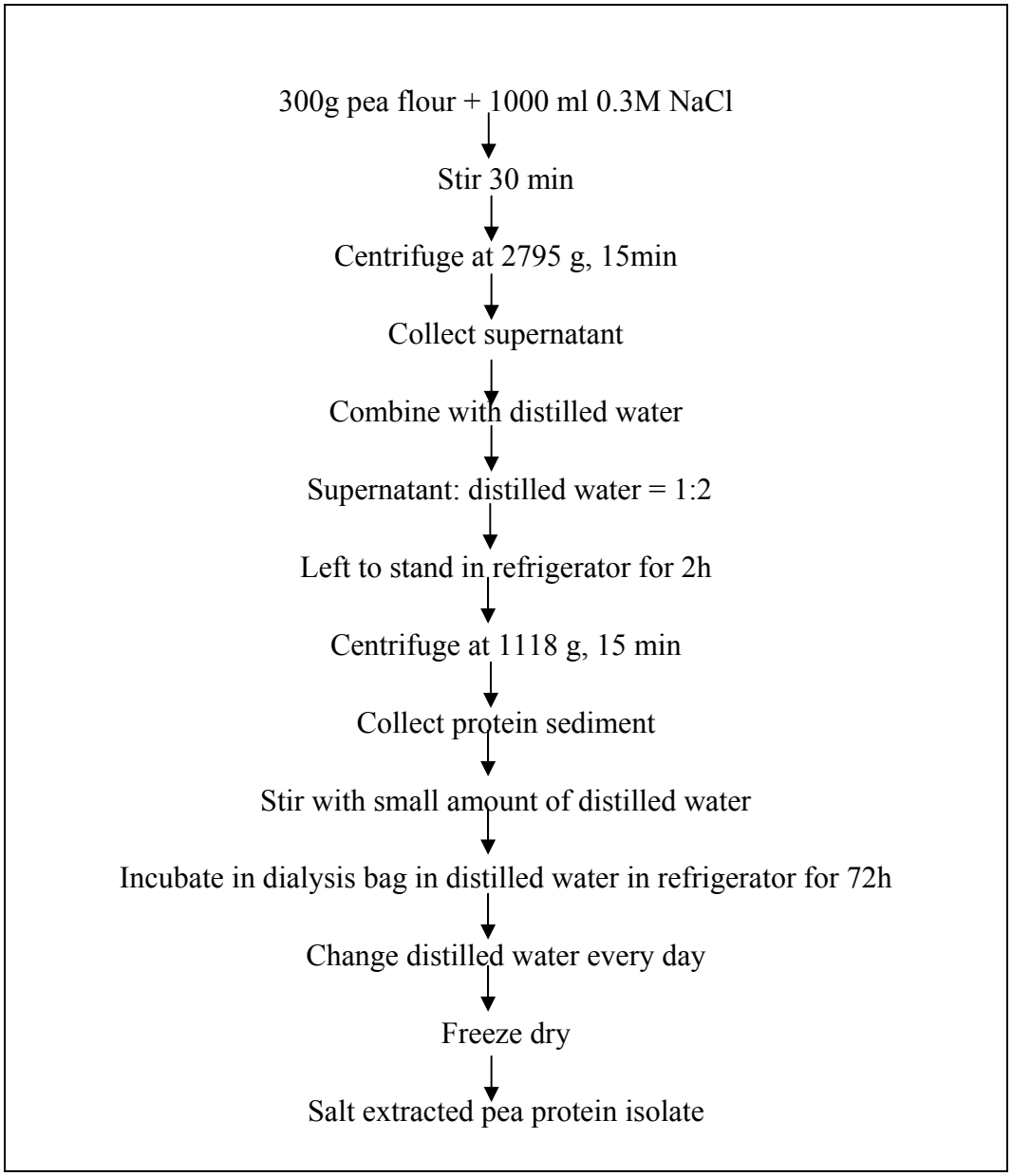

Fig. 3.1 Salt extraction pea protein procedure

3.3.3. Differential Scanning calorimetry (DSC)

The thermal properties of salt extracted pea protein isolate and commercial pea protein isolate suspensions were examined by a DSC Q200 (TA Instruments, New Castle, DE, USA). Instrumental conditions were as described in Shand et al. (2007). Peak transition temperature or denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed from the endothermic peaks observed in the thermograms using computer software (Universal Analysis 2000, Version 4.5A). In a typical experiment, 60-70 µL of a 10.5% protein sample was sealed in a preweighed stainless steel high volume pan and weighed again. An empty pan was used as reference. The sample was heated over a temperature range of 30-120 ºC in a standard DSC cell that had been calibrated with both indium and sapphire standards. Thermal curves were obtained at a heating rate of 10 ºC/min. Each sample was analyzed in duplicate.

3.3.4. Rheology

The pea protein isolate was mixed with 0.3M NaCl (Fisher Scientific, Ottawa, Canada) to obtain a suspension of the desired concentration. To achieve complete suspension, the samples were mixed by a Vortex-Genie Mixer (Scientific Industries Inc., Bohemia, N.Y., USA) for 1 min, then the sample was loaded to the rheometer (TA2000, TA Instruments, Newcastle, Del. USA). The pH of the suspension was between 5.65 and 5.70.

A TA2000 rheometer was used to test rheological properties of pea proteins. According to experimental protocol, approximately 1 mL of the pea protein isolate suspension was transferred to the lower plate of the parallel plate geometry. The upper

plate lowered down to give a gap width of 1.00 mm. To avoid water losses during measurement, a thin layer of light mineral oil was added to the well of the upper geometry and a solvent trap cover was used to prevent sample drying during heating. In this way, a water-saturated atmosphere was maintained at the surface of the sample.

The following heating protocol was used. Samples were first equilibrated at 25 °C for 2 min, then heated and cooled over a temperature range of 25-95-25°C at a controlled rate (4 °C /min, 2 °C /min, 1 °C /min, or 0.5 °C /min). Rheological data was collected during heating and cooling and during the frequency sweep every 10s. This was followed by a frequency sweep over a range of 0.01-10 Hz at 25 °C. Through this procedure, both changes during gel preparation and characteristic of the final gel were collected.

The storage modulus (G') and loss modulus (G") were determined as a function of frequency for each sample. The loss tangent or tan delta ($\tan \delta = G''/G'$), a measure of the energy lost due to viscous flow compared to the energy stored due to elastic deformation in a single deformation cycle, was also calculated. The input amplitude strain used for the dynamic analysis was 0.02, a value found to be in the linear viscoelastic region for heat induced protein networks in preliminary experimentation. Samples were run at least in duplicate.

The gelling point temperatures for the pea protein suspensions at different concentrations were determined by extrapolating the rapidly rising storage modulus G' during the initial heating phase to intercept the temperature axis.

3.3.5. Statistical analysis

All data were analyzed for significant differences, with minimum significance set at the 5% level ($P< 0.05$), using Tukey's test by GraphPad InStat software version 3.06 followed by analysis of variance (GraphPad Software Inc. La Jolla, CA, USA).

3.4. Results and discussion

3.4.1. Differential scanning calorimetry study of salt extracted and commercial pea protein isolates

The denaturation effects were evaluated by testing the impacts of NaCl concentrations on the thermal properties (T_d, ΔH) of pea protein isolates. NaCl concentration significantly affected the thermal denaturation properties of pea protein isolates (Table 3.1). The PPIs sample prepared in the presence of NaCl (0.3M) had higher thermal denaturation temperatures (T_d = 94.28 ºC) than the control containing no salt (T_d = 86.21 ºC). The actual heat flow into the macromolecules during the thermal denaturation process is described by the ΔH value. A greater heat flow is an indication that the pea protein was more native (less denatured) before the heat treatment. The PPIs samples in 0.3M NaCl had a higher ΔH values (17.84 J/g protein) than when no NaCl was included (15.81 J/g protein). These results show that NaCl stabilizes pea protein molecules against thermal denaturation (higher T_d and ΔH values). This was attributed to the presence of salt which stabilizes the quaternary structure of the protein against denaturation (Hermansson, 1986).

With the inclusion of low concentration NaCl, the increase in thermal denaturation temperatures can be attributed to nonspecific ion effects on electrostatic

interactions between charged groups on the protein. This induced the effect of stabilization of the globulin against thermal denaturation by anions. The stabilizing effects of NaCl could be attributed to two possible factors working separately or cooperatively. According to Damodaran (1988), these two factors are 1) charged side chains on the amino acid residues neutralized by NaCl thereby reducing inter and/or intra chain repulsion and 2) stabilization of water structure by salt. These ion-specific effects on hydrophobic interactions result from perturbations in the bulk water structure, which in turn influences segment–solvent and segment–segment interactions (von Hippel & Schleich, 1969). These ion-specific interactions are thought to modify proteins to more stable conformation such as an increase in α-helix or β-sheet and a decrease in random coil (Kwon, 1994).

Table 3.1 Effect of NaCl concentration on the thermal parameters (T_d, ΔH) of salt extracted and commercial pea protein isolate (10.5% w/v, heated at 10 ºC/min)

Protein sources	NaCl level (M)	T_d * (ºC)	ΔH * (J/g protein)
PPIs	0.0	86.21±0.05 [a]	15.81±0.03 [a]
	0.3	94.28±0.28 [b]	17.84±0.21 [b]
PPIc	0.0	72.92±0.042 [c]	0.036±0.01 [c]
	0.3	72.83±0.035 [c]	0.033±0.00 [c]

* Means±SD of duplicates.

[a~d] Column values followed by the same superscript letter are not significantly different (P<0.05).

In addition, it can be seen that T_d value of PPIs without salt (86.21 ºC) was higher than that of PPIc (72.92 ºC), and ΔH value of PPIs without salt (15.81J/g protein) was also greater than that of PPIc (0.036 J/g protein). In the study of Bora et al. (1994), it was found that the mixed globulins (35.7% legumin and 64.3% vicilin of pea) have one thermal transition with a T_d at 86.2 ºC. Shand et al. (2008) indicated that the commercial processes that are employed to isolate pea proteins tend to form a product rich in globulins, which is heterogenic and composed of legumins (11S) and vicilins (7S). In the present study, the lack of a transition peak at about 86 ºC for the PPIc indicated that the globulins in PPIc were completely denatured. Therefore, it appeared that the main protein fraction of globulins in PPIc was "seriously denatured" in the processing procedures which will influence its ability to form a gel.

It should be noted that the T_d values of PPIc were between 72.92 ºC (without salt) and 72.83 ºC (with salt) and were not statistically significantly (P<0.05) different. They were, however statistically lower than those of the corresponding PPIs samples (without salt - 86.21 ºC and with salt - 94.28 ºC). It is possible that the peak at approximately 73 ºC represented a thermal transition for something other than protein since the protein content of PPIc was only about 82%. As the commercial pea protein isolate was reported to contain starch by the supplier, this peak could represent starch gelatinzation. This was consistent with the result of Chavan et al. (1999). In their study the starches gelatinization temperatures T_p (mid-point) of green pea and grass pea were 72.0 ºC and 71.0 ºC, respectively. Jayakody et al. (2007) reported that the starch gelatinization temperature of two grass pea samples were 75.5 ºC and 73.3 ºC.

3.4.2. Typical gel formation pattern

A typical heat-induced gelation behaviour curve of pea protein is displayed in Fig. 3.2. The rheograms obtained from different measurements (replicates) were essentially identical; only one data set is presented.

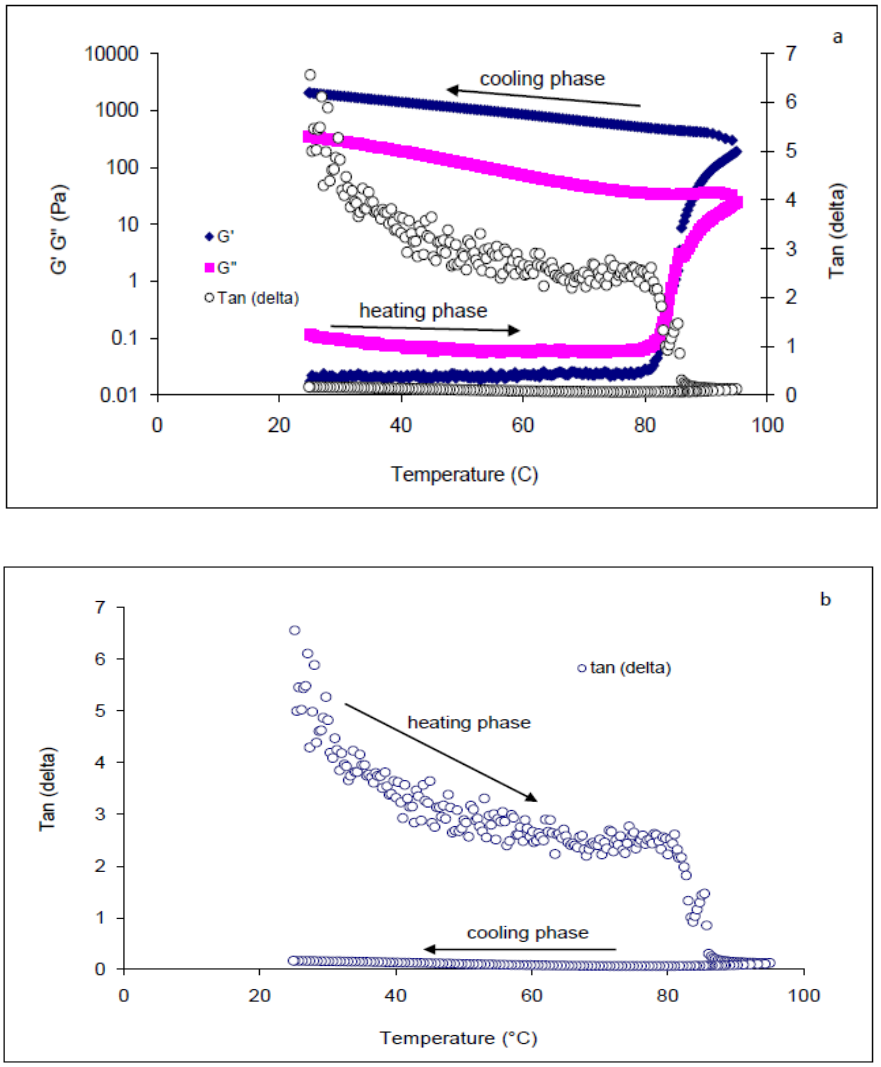

Fig. 3.2 A typical rheogram of pea protein gel formation.14.5% (w/v) salt extracted pea protein, 0.3M NaCl, 2 °C/min heating and cooling rate. (a) G' and G" pattern, (b) tan delta pattern.

Changes in rheological properties during network formation were seen for all three parameters (G', G", and tan δ). The G' or storage modulus is a measure of the elastic component of the network and represents the stiffness of the structure contributing to a three dimensional network. The G" or loss modulus is a measure of the viscous component and may represent interactions which do not contribute to the three dimensional nature of the network. As a result, a change in tan δ should be indicative of the type of network formed and lower tan δ values represent better three dimensional structures. As shown in Fig. 3.2a, it is clear that G' and G" values remained constant before the temperature increased to 80 °C in the heating phase. At this point the protein molecules denature and expose hydrophobic residues as a preparatory stage for gel formation. The gradual development of the network structure is reflected by a progressive increase in G' and G" at temperatures above 80 ºC. The beginning of the gelation process is dominated by the viscous behaviour of the system (G">G'), and the elastic behaviour dominated the final stages of heating (G'>G"), when protein molecules aggregate and crosslink to form a three-dimensional network. This was the initial stage of gelation. After reaching the maximum temperature of 95 °C, the cooling phase began. In this phase the G' and G" values continued to increase steadily until the end of the procedure. This indicated that the crosslinking continued with slower formation and rearrangement of the network structure. Paulson & Tung (1989) attributed the formation and stability of canola protein gels to the establishment of hydrophobic interactions and hydrogen bonds. Therefore, it could be assumed that the same interactions contribute to the formation and stability of pea protein gels. Tan δ is an important indicator to distinguish a gel formation. As seen in Fig. 3.2b, it decreased gradually before 80 °C, then plunged to a very low

level at ~ 85 °C, and remained constant at this low level, throughout the rest of the heating phase and the whole cooling stage. This suggested that a stable gel had formed from the beginning of cooling phase, and changes during the cooling phase added stiffness to both the elastic and viscous components of the network.

In a mechanism where aggregation is suppressed prior to unfolding, the resulting network can be expected to show lower opacity and higher elasticity than if random aggregation and denaturation occur simultaneously or if random aggregation occurs before denaturation (Hermansson, 1979). Tombs (1974) also concluded that the higher the randomness of aggregation the more likely it is that a coagulum is obtained instead of a gel.

In the present experiments, all the pea protein samples extracted by salt method had significantly higher G' than G" values when compared to the samples of commercial pea protein isolate (Table 3.2). As seen in Fig. 3.2, the gel formation of PPIs was initiated at or after the starting point of denaturation and G' values increased suddenly at the gelling point. Increasing rigidity of the gel with respect to heating and the strengthening of the gel network continued during cooling. This observation indicated that PPI followed the three-step process of gelation that is generally accepted for heat-induced gelation of such globular proteins. This process, as described by Clark et al. (2001), was summarized as follows: (1) denaturation of the protein with subsequent exposure of hydrophobic residures; (2) intermolecular hydrophobic interaction of the unfolded proteins (aggregation) and (3) agglomeration of aggregates into a network structure. It is important to note that in the cooling phase, the network continues to develop and is

strengthened by the formation of many short-range interactions such as hydrogen bonds (O'Kane et al., 2004a). O'Kane et al. (2004b) observed that network formation for the legumin proteins in pea and soy were mainly supported by hydrophobic and hydrogen bonds, while disulfide bonds had minimal involvement. Therefore, the increased exposure of hydrophobic residues on the protein molecular surface by heat denaturation probably induced gel formation. Mleko & Foegeding (2000) also ascribed the increase in storage and loss modulus of gels with an increase in temperature during heating to the presence of hydrophobic forces responsible for gelation. Therefore, it can be concluded that it was hydrophobic and hydrogen bonds that supported network formation from pea protein dispersion.

Table 3.2 Comparison of rheological properties of PPIs, PPIc, and SPIc. 14.5% protein dispersion, 0.3M NaCl, at 2 ºC/min heating and cooling rate, data were obtained at 1 Hz sweeping frequency.

	G' (Pa) *	G" (Pa) *	Tan δ *	Gelling point (°C)
PPIc	349.5±36.06 [a]	253.5±0.71 [a]	0.73±0.08 [a]	87.85±0.50
PPIs	3212.5±0.71 [b]	532.6±4.74 [b]	0.17±0.00 [b]	85.1±0.56
SPIc	889.5±44.55 [c]	225±5.66 [a]	0.25±0.02 [b]	NA

* Means±SD of duplicates.

[a~c] Column values followed by the same superscript letter are not significantly different (P<0.05).

3.4.3. Minimum gelation concentration

Clark & Ross-Murphy (1987) classified biopolymer gels based on the level of order of the macromolecule both before and during the network formation as (1) gels formed from disordered biopolymers, such as carrageenans, pectins, starch, and gelatin, and (2) gel networks that involve specific interactions between denser and less flexible particles, such as thermally denatured globular proteins and aggregated proteins from enzymatic or chemical action. In foods, the common method for globular protein gelation is heat-induced protein denaturation that causes changes in quaternary, tertiary, and secondary structures. It takes one globular protein molecule to denature, two or more to aggregate, and many more to form a gel matrix (Clark & Ross-Murphy, 1987). As a rule, to obtain gels from globular proteins requires concentrations an order of magnitude higher than, for example, from gelatin or gel forming carbohydrates (Tombs, 1974).

Thermal treatment is a physical procedure frequently used in the food industry to modify protein functionality (Boye et al., 1997). Isoelectric precipitation plus heat drying of pea proteins (commercial method) caused serious denaturation of the proteins compared to salt extraction combined with freeze drying. The most obvious evidence is the decreased gelation ability of acid precipitated pea proteins, supported by both physical observation and instrumental data. When the pea protein concentration was 14.5%, salt extracted pea protein isolate showed higher G' (3212.5 Pa) and lower tan δ values (0.17) swept at 1 Hz frequency compared to the G' (349.5 Pa) and tan δ value (0.73) of commercial pea protein isolate. Salt extracted pea protein isolate could form a good gel, whereas commercial pea protein isolate could only form a very weak gel.

In our study the minimum gelation concentration of salt extracted pea protein was 5.5%, and of commercial pea protein isolate was 14.5%. O'Kane et al. (2005) have reported that the minimum concentration required for heat-induced gel formation near neutral pH (pH 7.1) is 16% (w/v) for pea protein isolates containing 20–28% legumin and 61–67% vicilins in their composition. Because the PPI extraction method O'Kane used was isoelectric precipitation (pH 4.8), the same as commercial PPI, it caused serious denaturation of the pea protein and reduced its functionality. However, the minimum gelation concentration for PPIc (14.5%) is very close to O'Kane's (16%), since they both were prepared using an isoelectric precipitation method.

3.4.4. Gelling point

The gelling point is the temperature where a gel begins to form. It is widely accepted that the G'-G" crossover point is the gelling point (Winter, 1987). In this paper the gelling point is referred to the G'-G" crossover point following significant increase in G' and G" values.

It can be seen in Table 3.3 that gelling points of different concentrations of PPI dispersions fluctuated between 83 and 86 °C, but were not statistically significantly different (P<0.05), which means gelling point is concentration independent. It can be presumed from these data that formation of a protein network starting at about 80 °C led to appreciable mechanical properties for the gel.

Table 3.3 Influence of PPIs concentration on gelling point

	PPIs concentration							
	5.5%	7%	9%	10.5%	12%	14.5%	16%	18%
Gelling point (°C)*	84.5 ± 0.35 [a]	83.2 ± 1.13 [a]	85.7 ± 1.13 [a]	84.0 ± 1.41 [a]	85.6 ± 0.28 [a]	85.0 ± 0.56 [a]	85.6 ± 0.21 [a]	85.1 ± 0.14 [a]

* Means ± SD of duplicates.

[a] Row values followed by the same superscript letter are not significantly different (P<0.05).

3.4.5. Impact of heating and cooling rate

When heated at the fastest heating and cooling rate of 4 °C/min, the value of the storage modulus lagged behind those measured at the slower rates of 0.5 °C, 1°C, and 2 °C/min, therefore requiring higher temperatures to achieve the same degree of elasticity (Fig. 3.3). In addition, it can be seen that with slower heating and cooling rate, the G' values observed during the cooling phase for 0.5 °C/min were the greatest and those of 4 °C/min were the smallest. Slower heating and cooling rates enable more protein molecules to form elastic strands in the gel network and protein molecules also have more time to rearrange in a certain order which strengthens the gel.

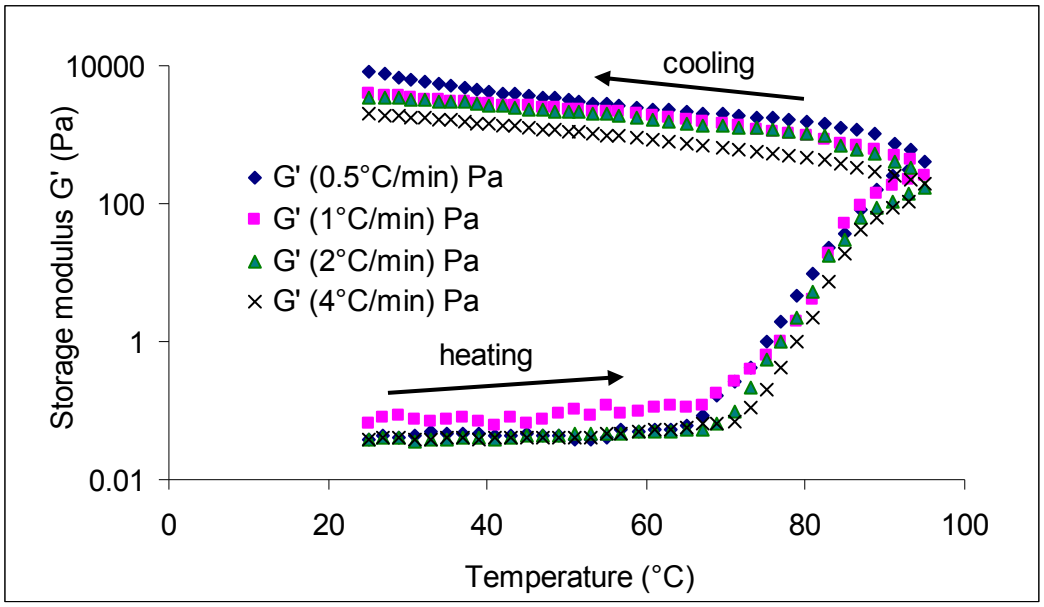

Fig. 3.3 Impact of heating and cooling rate on development of storage modulus G'. 14.5% (w/v) PPIs, 0.3M NaCl.

As for the impact of heating rate on gelling point, there is a tendency that with increasing heating rate, the gelling point also increased (Table 3.4). However, at higher heating rate (2 to 4 ℃/min), this tendency was inconsistent because the gelling point was not significantly affected. Therefore it can be concluded that at lower heating rates (0.5 or 1.0 ℃/min) the gelling point was heating rate dependent, whereas at higher heating rates the gelling point was independent of heating rate. At a lower heating rate the protein molecules have more time to rearrange and align thus they begin to crosslink earlier at lower temperature, whereas at a higher heating rate, protein molecules do not have sufficient time to rearrange and align, therefore they start to crosslink later. At a heating rate of 2 °C/min and greater, the rearrangement and alignment time were less variable as the gelling point fluctuated between 83 and 85 °C.

Table 3.4 Influence of heating and cooling rate on gelling point

	0.5 °C/min	1 °C/min	2 °C/min	3 °C/min	4 °C/min
Gelling point (°C) *	61.1±1.8 [a]	68.1±1.9 [b]	85.0±0.56 [c]	83.6±0.2 [c]	84.3±1.4 [c]

* Means ± SD of duplicates.

[a~c] Row values followed by the same superscript letter are not significantly different (P<0.05).

3.4.6. Impact of protein concentration

Effect of protein concentration on G' and G" are shown in Fig. 3.4. It can be seen that the storage modulus G' and loss modulus G" increase with increased PPI concentration (in the range of 4-18%), due to the increase in opportunities for cross-linking of PPI. Therefore, it can be concluded that higher PPI concentrations induce formation of stronger gels.

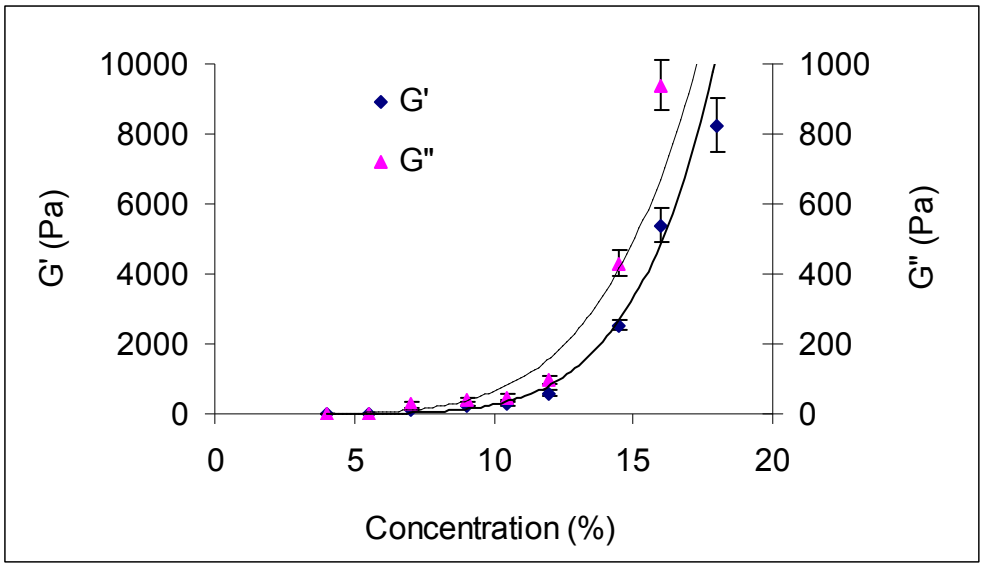

Fig. 3.4 Relationship between pea protein concentration and the G' and G" moduli for heat induced networks, PPIs, 0.3M NaCl. Error bars represent standard deviation.

A power law relationship between G' and concentration or G" and concentration was obtained for pea protein. In the present study, the following equations were obtained:

$G' = 0.0002(C)^{6.2}$; $R^2 = 0.9677$

$G'' = 0.0006(C)^{5.0}$; $R^2 = 0.9664$

Where C represents the pea protein concentration.

By using these power law equations, G' and G" values could be predicted when selecting a protein concentration. This enable us to attain desired G' and G" values by adjusting pea protein concentration.

The gelation process can also be monitored by measuring changes in tan δ values during dynamic testing. The ratio G"/G', i.e., tan δ, has been reported to be approximately 0.01 for a solid gel and 0.1 for weak gel (Ross-Murphy, 1984) using oscillatory rheology at low frequencies. Relatively low tan δ values for gels represent elastic structures. It was shown in Fig. 3.5 that tan δ value decreased rapidly from 0.98 to 0.17 when PPI concentration increased from 4% to 7%, then tan δ values fluctuated between 0.16 and 0.17, which indicated that weak gels formed when PPI concentration was greater than 5%.

3.4.7. Frequency sweep of PPI

Generally, G' increased slightly with the increase in oscillatory frequency (Fig. 3.6). For a totally elastic system, the G' values would be independent of frequency. Therefore, the slight dependence on frequency observed with the gel networks reflected the viscoelastic contribution. The frequency sweep test of the gel formed after heating and cooling provides information on the final gel properties (Cai & Arntfield, 1997).

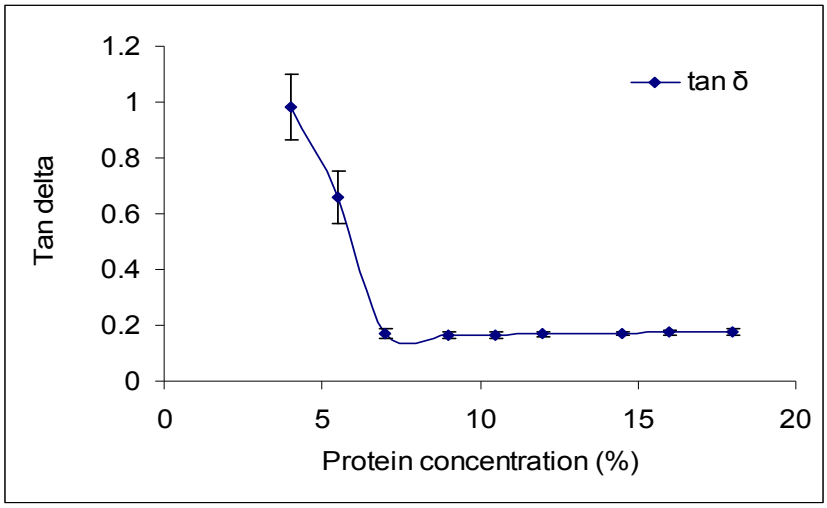

Fig. 3.5 Effect of protein concentration on tan δ for heat induced networks. PPIs, 0.3M NaCl. Error bars represent standard deviation.

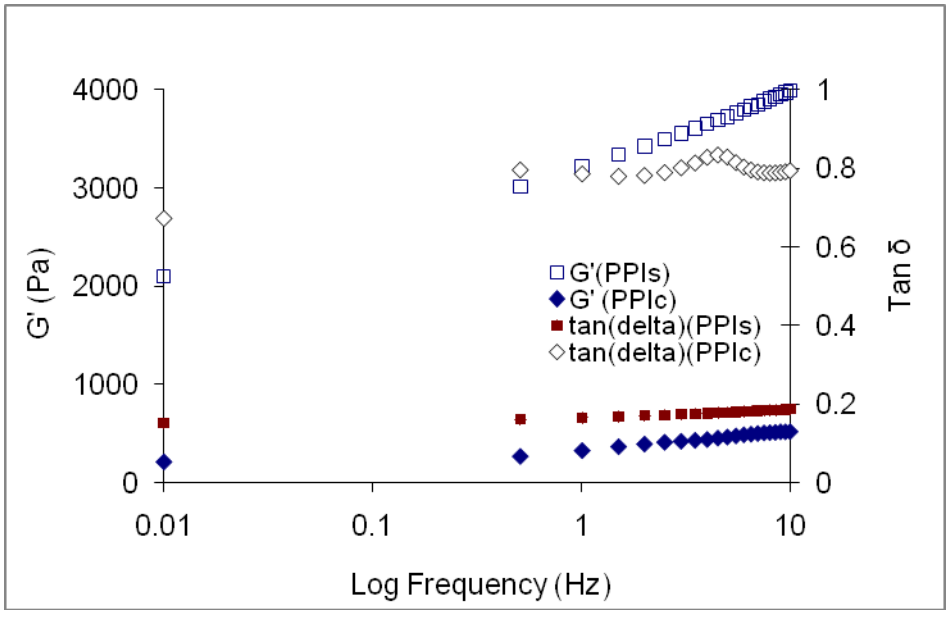

Fig. 3.6 Comparison of gelation properties of commercial and salt extracted PPI. 14.5% (w/v) pea protein concentration, 0.3M NaCl.

As seen in Fig. 3.6 the shapes of curves representing the G' and tan δ versus frequency (ω) for salt extracted and commercial PPI were slightly different. The salt extracted protein had higher G' and lower tan δ values. According to Clark & Ross-Murphy (1987), in a strong gel, the G' is higher than G" throughout the frequency range, and G' is almost independent of frequency. In weak gels, there is a higher dependence on frequency for the dynamic moduli, and lower difference between moduli. Based on the lower G' values and higher tan δ values, it would appear the PPIs formed a weak gel, but it was stronger than that formed by the commercial pea protein isolate.

3.4.8. Comparison of PPIs, PPIc, and SPIc

To verify if PPIs could form a better gel in comparison with PPIc, gels of PPIs and PPIc made under the same conditions are compared in Fig. 3.7.

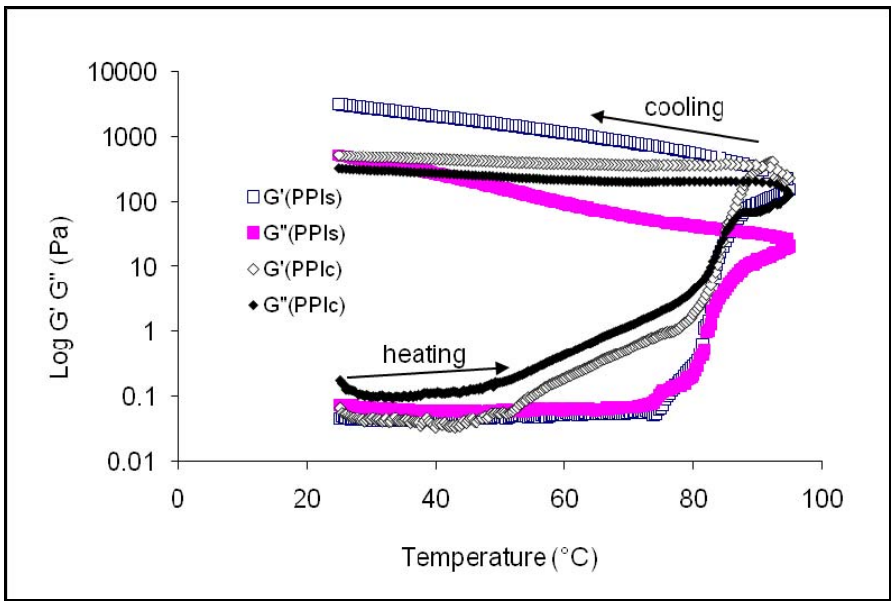

Fig. 3.7 Comparison of heating and cooling phase of salt extracted and commercial PPI. Pea protein concentration was 14.5% (w/v) in 0.3 M NaCl.

In Fig. 3.7 we can see that for both PPIs and PPIc, G' values are greater than those of G" during cooling phase as would be expected during gel formation. However, the final G' and G" values are much greater in salt extracted PPI than in commercial PPI. This could be related to the level of pea protein denaturation during processing. As the commercial PPI was extracted using isoelectric precipitation and spray drying, it may have undergone some denaturation for the following two reasons. First, the isoelectric precipitation method uses NaOH to extract the protein, the pH is then adjusted to 4.5 (isoelectric point) to precipitate the protein. The recovered protein is dispersed in water and neutralized to pH 7 with NaOH. The initial NaOH extraction is likely to denature the protein. Second, spray drying is a heat drying method that may also contribute to pea protein denaturation. The fact that no endothermic peak for protein (only a small starch peak was seen) was detected when analyzed using DSC (Table 1) confirms that the protein has been denatured during preparation. The denatured commercial PPI, therefore, results in the formation of a weakened gel structure. The salt extraction method was milder and uses freeze drying, therefore caused less denaturation (ΔH =15.81 J/g protein in DSC), resulting in stronger gelation properties than the commercial PPI. Fig. 3.7 illustrates the gel firming that occurred during the cooling phase from 95°C to 25°C. In this phase both G' and G" increased steadily for the PPIs suggesting a fine network is forming. In addition, the tan δ value (0.17) for PPIs was considerably smaller than that of PPIc (0.73). The gelling point of PPIc of 87.5 ºC was higher than that for PPIs (85.1 ºC - Table 3.2). All these parameters indicate that PPIs formed a finer and stronger gel than that of PPIc.

From the above comparison, we can draw the conclusion that PPIs could form a stronger gel than that of PPIc. As the SPIc was also extracted by isoelectric precipitation and spray drying, it is expected that this procedure would induce denaturation of soy protein to some extent and would affect its gelation properties. As seen in Table 3.2, the G' value of SPIc only reached 889.5 Pa, a much smaller value than that of PPIs (3212 Pa). Meanwhile, the tan δ value of SPIc was 0.25, higher than the 0.17 obtained for PPIs. These parameters indicate that the gel stiffness of PPIs should be stronger than that of SPIc. However, based on visual observation of the gels it appeared that the PPIs gel was weaker than that of SPIc. O'Kane et al. (2004b) and Soral-Śmietana et al. (1998) found that soybean glycinin was able to form a better network than pea legumin (possibly due to the availability of lysl and glutaminyl residues) based on the textural properties of the heat-induced gels. In this case the rheometer data did not show satisfactory relationship with the actual observation; this suggests that small amplitude oscillatory tests such as that used in this study may need to be combined with other tests such as large deformation texture analysis to better reflect the observed gel properties.

As shown in Table 3.2, there was no gelling point data for SPIc. The reason for this is because at the beginning of the rheological test, the G' values were already greater than those of G". In other words, there was no G'-G" crossover point during the heating phase. This indicated that SPIc showed more elastic properties than that of PPIs and PPIc prior to heating.

3.5. Conclusion

Pea protein can form heat-induced weak gels. The gelation of pea protein is temperature-dependent, and primarily influenced by the degree of protein denaturation. If the degree of denaturation is lower, a stronger gel is formed. Protein concentration also plays an important role in gelation properties, and generally higher concentrations induce stronger gels. However, the gelling point is partially concentration independent. Heating and cooling rates were minor factors influencing gelation properties of pea protein. It was shown that heating rate influenced the gelling point in that higher heating rates resulted in delayed gelling temperatures. Higher heating and cooling rates caused a weakening effect of gel elasticity as evidence by lower final G' values. In conclusion, salt extraction is a mild method causing minimal protein denaturation. PPI extracted using this method showed superior gelation ability in comparison with acid extracted (commercial) sample and has great prospects as a functional protein additive in food products.

Chapter 4: Heat-induced gelation of pea protein extracted by salt and precipitation by dilution: Effect of pH and NaCl

4.1. Abstract

The effects of two important factors, pH (3.0~10.0) and salt (0~2.0M), on pea protein gelation properties were studied using rheometer and differential scanning calorimeter. The stiffest gel was achieved at 0.3M NaCl. Higher or lower salt concentration led to weakening of the gel. The gelation temperature was also influenced by salt. Salt had a stabilization effect which inhibited pea protein denaturation at higher salt level resulting in higher gelling points. At a NaCl concentration of 2.0 M, pea protein gelation was completely suppressed at temperatures ≤ 100 °C. The pH also played an important role in gel formation by pea protein isolates since acid and base cause partial or even total protein denaturation. The maximum gel stiffness of PPIs at 14.5% (w/v) occurred at pH 4.0 in 0.3M NaCl. Higher or lower pH values resulted in reduced gel stiffness. pH also altered the denaturation temperature of the pea protein; higher pH values resulted in higher denaturation temperatures and higher enthalpies of denaturation. At pH 3, pea proteins seemed to be completely denatured by acid as the DSC curve showed a straight line. The gelation temperature (gelling point) peaked at ~ pH 6.0 (89.1 ºC). Careful adjustment of pH and NaCl concentration would enable the food industry to effectively utilize the salt-extracted pea protein isolate as a gelling agent.

4.2. Introduction

Protein gels can be divided into two types, gels formed by "random" aggregation and gels formed by association of molecules into strands in a more ordered way (Hermansson, 1986). Due to small changes in the repulsive balance, gels of both types can be formed from one protein and the transition from one type of gel structure to another can take place within 0.1 pH units (Hermansson, 1986).

It has been proposed that electrostatic forces are involved in the gelation of whole plasma protein since the gel formation is affected by pH and salts (Hickson et al., 1980; Hermansson, 1982a, b; O'Riordan et al., 1988a, b); therefore, electrostatic interactions are also expected to be involved in the gelation process of pea protein. As a result, the inclusion of salts, which affect electrostatic interactions, would be expected to influence pea protein gelation.

Usually heat denaturation is a prerequisite for gel formation of globular proteins. Denaturation temperatures depend strongly upon pH and salt concentration (Hermansson, 1986). A number of studies have investigated the effect of salt (ionic strength) and pH on gelation properties of both muscle and plant proteins (Chang et al., 2001; Lakemond et al., 2003; Ma et al., 1988; Puyol et al., 2001; Renkema et al., 2000; Uruakpa & Aluko, 2004; Westphalen et al., 2005) and it was shown that the gelation properties of both were affected by salt and pH. Stronger gels were formed at adequate salt concentration (0.3~0.6 M NaCl) and in the acidic pH range (pH 3~6). Shand et al. (2007) investigated the effect of pH and salt on commercial pea protein isolate gel formation and found that the optimal condition for a strong heat-induced gel was 2% (0.34 M) NaCl at pH 7.1. A

salt extraction method was developed to obtain low denaturation pea protein isolate due to weak gel forming ability of commercial pea protein isolate (Sun & Arntfield, 2010). The effects of the combination of pH and salt concentration on gelation properties of salt extracted pea protein isolate have not been extensively studied. This study is focused on the effect of pH on gelation characteristics of salt-extracted pea protein isoalte in relation to denaturation at different salt concentrations.

4.3. Materials and methods

4.3.1. Commercial pea flour, PPIs

Commercial pea flour and pea protein isolate were donated by Nutri-Pea Ltd (Portage la Prairie, MB, Canada). The Century Flour was made from Canadian yellow pea by a wet milling process; protein content was greater than 25%, carbohydrate content less than 75%, and starch content around 50%. The commercial pea protein isolate (PPIc) —Propulse contained 82% protein, less than 12% carbohydrate with less than 0.7% starch as reported by the supplier. Commercial soy protein isolate (SPIc) (PRO-FAM® 974 soy protein) was obtained from Archer Daniels Midland Company (ADM) (Decatur, IL, U.S.A.) with a reported protein content of 90%, and total carbohydrate content less than 1%. The pea protein isolate prepared by salt extraction, with dilution by precipitation was described previously (Sun & Arntfield, 2010).

4.3.2 Preparation of pea protein dispersion

Prior to analysis, the pea proteins (14.5%, w/v) were suspended in 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1.0, and 2.0M NaCl solutions. By adding 2M HCl or 2M NaOH, pea protein dispersions with different NaCl concentration were adjusted to pH 3~11 using an

Accumet AB15^{+} Basic pH meter (Fisher Scientific, Ottawa, Canada) and monitoring pH until it stabilized at the desired pH.

4.3.3. Differential Scanning calorimetry (DSC)

The thermal properties of salt extracted pea protein isolate suspensions were examined using a DSC Q200 (TA Instruments, New Castle, DE, USA). Instrumental conditions were as described in Shand et al. (2007). Peak transition temperature or denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed from the endothermic peaks observed in the thermograms. In a typical experiment, 60-70 µL of the 10.5% protein sample was sealed in a preweighed stainless steel high volume pan and weighed again to determine sample weight. An empty pan was used as reference. The sample was heated over a temperature range of 30-140 ºC in a standard DSC cell that had been calibrated with both indium and sapphire standards. Thermal curves were obtained at a heating rate of 10 ºC/min. ΔH values were reported in J/g protein. Each sample was analyzed in duplicate.

4.3.4. Rheology

Rheological parameters were determined as described in Sun & Arntfield (2010).

4.3.5. Gelling temperature (point)

The gelling point temperatures for the pea protein suspensions at different concentrations were determined as the G'-G" crossover point (Winter, 1987; Friedrich & Heymann, 1988; Clark, 1991; Ikeda & Nishinari, 2001)

4.3.6. Statistical analysis

All data were analyzed using analysis of variance (GraphPad Software Inc. La Jolla, CA, USA). Significant differences, with minimum significance set at the 5% level ($P<0.05$), were identified using Tukey's test with GraphPad InStat software version 3.06.

4.4. Results and discussion

4.4.1. Effect of pH on gelation properties of PPIs

In chapter 3 we demonstrated that a protein concentration of 14.5%, 0.3 M NaCl at natural pH (5.65) was necessary for gel formation using pea protein isolate. Therefore, we have used 14.5% protein to study the effect of pH on gelation properties. A salt concentration of 0.3 M was chosen to evaluate a wide pH range. The G' values for gels prepared from pea protein isolate were dependent on pH (Fig. 4.1). The G' – pH curve had increased values at both acidic (pH 4) and alkaline (pH 10) values, respectively. This is consistent with the observation of Egelandsdal (1980), who also found stronger ovalbumin gels formed at acidic and alkaline pH values, respectively. From the above results it would appear that at pH 4, heating conditions (95 °C) were sufficient to achieve denaturation, resulting in increased protein-protein interaction and the highest G' value. At pH values between 6 and 8, the denaturation temperatures were above 95 °C (Table 4.1) and as a result the heating regime would not be sufficient to promote pea protein (vicilin and legumin) denaturation. This is because the globular structure of pea proteins were not adequately opened and the buried functional groups were not available for crosslinking, therefore, the interactions necessary to increase the G' value did not occur. The slight increase in G' at pH 10 may be due to pH induced denaturation rather

than heat denaturation. Meng & Ma (2001) suggested that at high or low pH values, net charge as well as repulsive forces are increased, resulting in an unfolding of protein molecules. Especially at high pH levels, the repulsive force of protein molecules are so high that it influences the gel structure and induces a decrease in the gel strength. Our results are consistent with their findings.

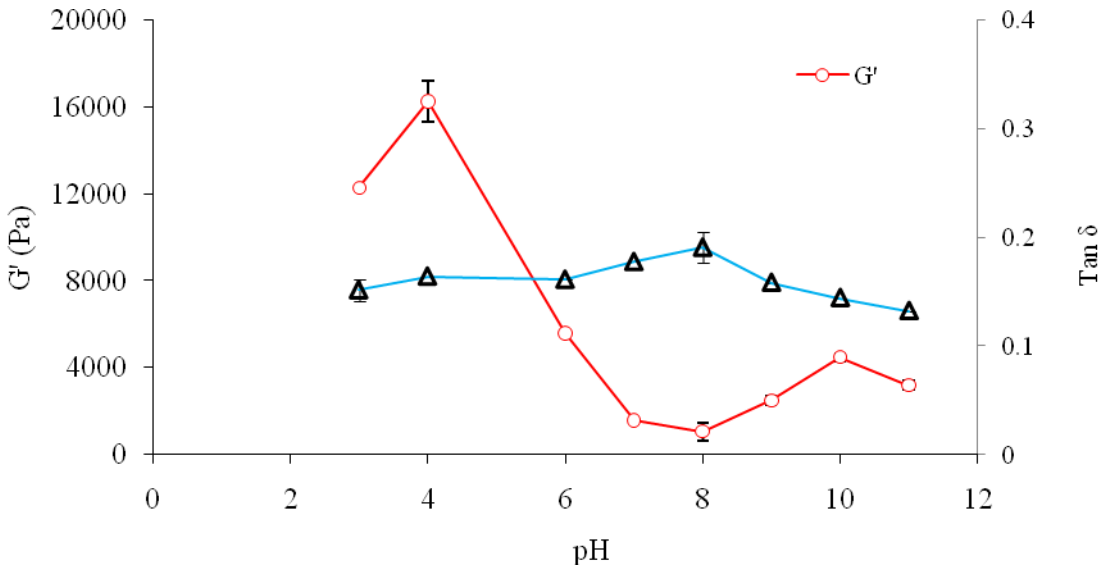

Fig. 4.1 Effect of pH on storage modulus G' and tan δ. PPIs concentration of 14.5% (w/v), 0.3 M NaCl were used. Error bars represent standard deviations.

In Fig. 4.1 we can see that pH – tan δ curve also showed high points at both acidic and alkaline pH with maximum values at pH 4 and 10. As tan δ values represent the G"/G' value, a low tan δ value indicates a stronger role for G' in the network formed and can represent the relative elasticity of the network. As a result, the lower tan δ values around pH 5 and 10 would suggest better networks. The higher tan δ, higher G'

combination at pH 3~4 could indicate aggregation rather than network formation, while the low G', high tan δ values from pH 7 to 9 would suggest very little network formation. The increase in G' at pH 10 may be due to pH induced denaturation rather than heat denaturation as the denaturation temperature was still above 95 ºC.

Table 4.1 Effect of pH on gelling point and thermal parameters (T_d, ΔH) of salt extracted pea protein isolate

pH	3	4	5	6	7	8	9	10	11
Gelling point (°C) *	55.9 ± 0.8	75.2 ± 0.7	NA **	89.1 ± 0.1	86.5 ± 0.9	81.9 ± 2.1	70.4 ± 0.5	59.2 ± 0.1	85.1 ± 0.1
T_d (°C)	–	83.2 ± 1.5	94.4 ± 0.4	94.5 ± 0.1	96.6 ± 0.3	96.6 ± 0.3	95.9 ± 0.2	95.8 ± 0.8	94.2 ± 0.6
ΔH (J/g)	–	7.4 ± 0.4	13.6 ± 0.1	16.2 ± 0.6	17.8 ± 0.3	16.8 ± 0.5	15.8 ± 1.2	14.9 ± 1.3	9.5 ± 0.6

For gelling point: pea protein concentration 14.5%, w/v, 0.3M NaCl, heating and cooling rate 2 ºC/min. For thermal parameters: pea protein concentration 10%, w/v, 0.3 M NaCl, heated at 10 °C/min.

* Means ± SD of duplicates.

** NA, not available at this pH value due to low denaturation of pea protein.

4.4.2. Effect of pH on gelling temperature (point)

As seen in Table 4.1, the gelling point was highest at pH 6. The gelling point decreased very quickly when pH decreased in the acidic range, but a slower decrease in gelling point with pH was observed in the alkaline range. This could be explained by the acid or alkali denaturation of pea protein. This hypothesis was supported, in part by the DSC data at various pH values in 0.3 M NaCl (Table 4.1). The highest denaturation

temperature (T_d) and enthalpy (ΔH) of denaturation were seen at pH 7~8 (T_d = 96.6 ºC; ΔH = 16.8~17.8 J/g protein). The decrease in those values was much greater under acidic conditions (at pH 3, T_d = 83.2 ºC; ΔH = 7.4 J/g protein) than alkaline conditions (pH 10, T_d = 95.8 ºC; ΔH = 14.9 J/g protein). No DSC transition was detected at pH 3 (DSC curve showed a straight line), indicating that the pea proteins were completely denatured. Due to low solubility of pea proteins at pH 5, it was impossible to run a sample on rheometer. At pH 11, this trend was reversed and the gelling point was higher than at pH 10. This is probably due to the very high alkali concentration creating a negatively charged molecule and the strong repulsive force inhibited gel formation or there is some hydrolysis at this very alkaline pH. This point of view is supported by the results of Meng & Ma (2001). In addition, the lowered T_d and ΔH values at pH 11 in comparison with pH 10 could be attributed to alkaline hydrolysis.

4.4.3. Effect of NaCl on gelation properties of PPIs

It can be seen that the gelling point increased with increasing salt concentration (Table 4.2); however, at the salt concentration of 2.0 M NaCl, there was no evidence of the G'/G" crossover during heating to 95 °C. This was attributed to the presence of salt which stabilized the quaternary structure of the protein against denaturation and at 2.0 M, gelation was completely suppressed at ≤ 100 °C (Hermansson, 1986). As seen in Table 4.2, the G' value of formed gel without adding NaCl (0M) was very small (G' = 0.35, tan δ= 1.36). This low G' may be due to aggregation of protein molecules since without salt, pea protein solubility is low and the suspension is unstable, and can easily to be denatured by heating and the denatured proteins would aggregate. Due to severe aggregation, pea protein isolate was more likely to form macroaggregates which are

unfavorable for gel formation. Another explanation is that electrostatic repulsion between proteins prevented gel formation. Studies on the various forces involved in network formation and their influence on rheological properties of milk β-Lactoglobulin (β-Lg) protein gels concluded that excessive repulsive forces created a high enough energy barrier to prevent denatured protein molecules from associating to form a strong self-supporting gels when NaCl was not added (Mulvihill & Kinsella, 1988; Mulvihill et al., 1990, 1991).

Table 4.2 Influence of NaCl on gelation properties of salt extracted pea protein isolate (pea protein concentration 14.5%, natural pH 5.65) at 1.0 Hz frequency

	G' (Pa) *	G'' (Pa) *	Tan δ *	Gel point (°C) *
0 M	0.35±0.17	0.42±0.01	1.36±0.65	60.15±1.91
0.1M	0.61±0.23	0.05±0.05	0.13±0.03	65.45±1.20
0.2M	791±63.64	114.50±12. 02	0.145±0.00	78.90±0.42
0.3M	4516±188	757±31	0.168±0.00	88.8±0.21
0.4M	3672.5±99.70	610.70±22.20	0.165±0.00	86.65±0.64
0.5M	2991.5±26.16	483.45±10.96	0.162±0.00	87.40±0.00
0.6M	3454±159.8	567±6.72	0.164±0.00	88.7±0.14
0.7M	3857±93.34	660.45±11.95	0.171±0.00	88.8±0.42
0.8M	3139.5±180.31	574.25±54.66	0.183±0.01	90.35±0.07
0.9M	2574.5±45.96	439.5±7.78	0.171±0.00	91.2±0.14g
1.0M	2204±79.90	387.2±5.94	0.175±0.00	92.75±0.07
1.5M	972±77.78	212±33.94	0.218±0.02	93.65±0.35
2.0M	0.38±0.11	0.82±0.03	2.25±0.55	NA

*Means ± SD of duplicates.

Higher G' values were seen between 0.3 and 0.8 M NaCl with the highesst achieved at 0.3 M NaCl, a value significantly higher than at other salt concentrations (Table 4.2). At salt concentration other than 0.1M, tan δ values were greater than 0.14 and pea protein solution formed a coagulum on the rheometer. Although large differences in tan δ were seen between 0.1 and 1.5 M, the high variability in the samples with 0 or 2 M salt made it difficult to pick out small differences.

It is noteworthy that although gelling temperature increased with increasing salt concentration, this had no impact on gel stiffness. Without salt, the G' value was very low, only 0.35 Pa, and tan δ value was high (1.36, Table 4.2). This indicated a very weak gel (or no gel) compared to those formed at high salt concentration (0.3 M~1.0 M NaCl). Although G' was only 0.61 Pa for 0.1 M salt, tan δ value decreased to 0.13, and while there was aggregation, syneresis was observed for this system. This could be explained by the study of Castimpoolas & Meyer (1970) using soy protein where they attributed the initial increase in G' to a shielding effect of salt (up to 0.2 M) on the surface charges, which in turn led to a reduction in the repulsive forces between protein molecules. Further increases in salt concentration have been shown to negatively influence gel formation by decreasing protein unfolding (Boye et al., 1995). This may account for the decrease in G' seen at NaCl concentrations above 0.8 M. When NaCl concentrations were equal to or greater than 1.0 M, the effects on gelation properties could also be due to salting out properties.

When using 2.0 M NaCl, the G' value was again very low (0.38 Pa). In fact, G' was lower than G" (0.82 Pa) resulting in a tan δ value of 2.26, indicating that the PPIs

could not form a gel. Thus no G'-G" crossover point showed up. The PPIs dispersion at this high salt concentration remained in solution at the end of the experiment because heating temperature of 95 ºC was too low to reach the denaturation temperature of salt stabilized pea protein of ~ 117 ºC (Table 4.3).

Table 4.3 Effect of pH and salt concentration on denaturation temperature (T_d) of salt extracted pea protein isolate (10% w/v, heated at 10 °C/min)

NaCl	pH*				
mol/L	4	5	6	7	8
0	84.28±0.42	88.02±0.13	86.70±0.15	81.31±0.30	79.98±0.09
0.3	83.20±1.50	94.40±0.40	94.50±0.10	96.60±0.30	96.60±0.30
0.5	90.13±0.35	97.13±0.25	98.77±0.09	99.03±0.01	99.44±0.77
1.0	96.39±0.19	105.08±0.09	107.66±0.01	107.35±0.52	105.81±0.42
1.5	102.80±0.64	109.65±0.31	111.68±0.46	112.19±0.52	110.83±0.36
2.0	108.12±0.48	114.56±0.01	117.41±0.85	117.67±0.76	116.97±0.33

* Mean ± SD of duplicates.

The strongest (highest G') and most elastic (lowest tan δ) gels were found between 0.3 and 0.8 M NaCl as the balance between attractive and repulsive forces was suitable for gel formation. At higher salt concentrations G' gradually decreased. Hermansson & Akesson (1975) have suggested that at higher salt concentrations, an increase in protein-solvent interaction could weaken protein gels. Our results are consistent with their point of view.

In emulsified meat products, such as frankfurters and bologna, the salt (NaC1) concentration is approximately 2.5%. This percentage would correspond to

approximately 0.4 M NaCl. Thus, the use of pea protein in emulsified meat products at about 0.3 ~ 0.4 M NaCl level should produce an almost optimum salt level that both meat and pea protein form stable gels.

4.4.4. Interaction affects involving pH and NaCl

In addition to the independent effects of pH and NaCl, there was an interaction between these two parameters for both the thermal (Table 4.3 and Table 4.4) and rheological data (Fig. 4.2). While T_d values followed a similar trend when NaCl was included, the response was quite different when no salt was included. With no salt, the T_d value was highest at pH 5 to 6 and decreased at both acid and alkaline pH values. This could reflect the denaturation of the proteins at pH values below and above the isoelectric point. When salt was included the T_d value was lowest at pH 4, and was much higher at pH 5 than pH 4 for all salt levels. The T_d values between pH 6 and 8 were higher at pH 5, but were not significantly different from each other. While similar behaviour was seen for all salt concentrations, the effect of salt concentration was apparent as higher salt levels resulted in higher T_d values (Table 4.1). The increase in protein stability due to the inclusion of salt was stronger than the denaturing effect of high pH seen when no salt was present, except at pH 4 between 0 and 0.3 M NaCl, increased salt concentrations resulted in significant increase in T_d values at all pH levels. This trend was consistent with the result of Shand et al. (2007), although they only examined commercial pea protein isolate slurries between pH 6.4-6.5, at NaCl concentrations of 0%, 1% (0.17 M), and 2% (0.34 M). Renkema et al. (2000) compared T_d values of soy protein isolate at pH 3.8 and 7.6

and illustrated that T_d decreased with decreased pH. Our results are also in agreement with their observations.

Table 4.4 Effect of pH and salt concentration on enthalpy of denaturation (ΔH) of salt extracted pea protein isolate (10% w/v, heated at 10 °C/min)

NaCl	pH*				
	4	5	6	7	8
0	8.31±0.97^{aA}	11.4±1.33^{bA}	15.38±0.71^{cA}	12.81±0.06^{bA}	11.58±0.23^{bA}
0.3	7.40±0.40^{aA}	13.60±0.10^{bA}	16.20±0.60^{cA}	17.80±0.30^{cA}	16.80±0.50^{cA}
0.5	9.23±0.12^{aB}	14.34±0.64^{bA}	16.12±0.21^{bA}	16.71±2.19^{bA}	15.82±0.23^{bA}
1.0	9.19±0.09^{aB}	14.04±1.10^{bA}	14.53±0.54^{bA}	14.64±0.07^{bA}	15.17±0.93^{bA}
1.5	8.70±0.52^{aA}	11.19±1.03^{abA}	15.62±1.65^{bA}	15.99±2.62^{bA}	15.89±2.08^{bA}
2.0	8.62±0.33^{aA}	13.02±0.02^{bA}	14.79±0.59^{bA}	15.41±0.74^{bA}	13.37±1.19^{bA}

*Mean ± SD of duplicates.

$^{a\sim d}$ Row values followed by the same superscript lowercase letter are not significantly different ($p<0.05$).

$^{A\sim B}$ Column values followed by the same superscript capital letter are not significantly different ($p<0.05$).

The changes in enthalpy (ΔH) due to interaction between NaCl concentration and pH followed a similar trend. In the absence of salt, the ΔH value was highest at pH 6 and was lower at both alkaline and acid pH values due to prior denaturation of the protein. When salt was included, the lowest ΔH values were at pH 4, while values between pH 5 and 8 were not significantly different. The effect of salt concentration only distinguished between the presence or absence of salt at alkaline pH values (7 and 8). There were no differences in ΔH values between 0.3 and 2 M NaCl for any of the pH values examined. In a study by Shand et al. (2007), where they investigated ΔH values of pea protein isolate slurries at single pH 6.4-6.5, salt concentrations of 0%, 1% and 2% had no significant effect on ΔH values. Our results are consistent with their finding.

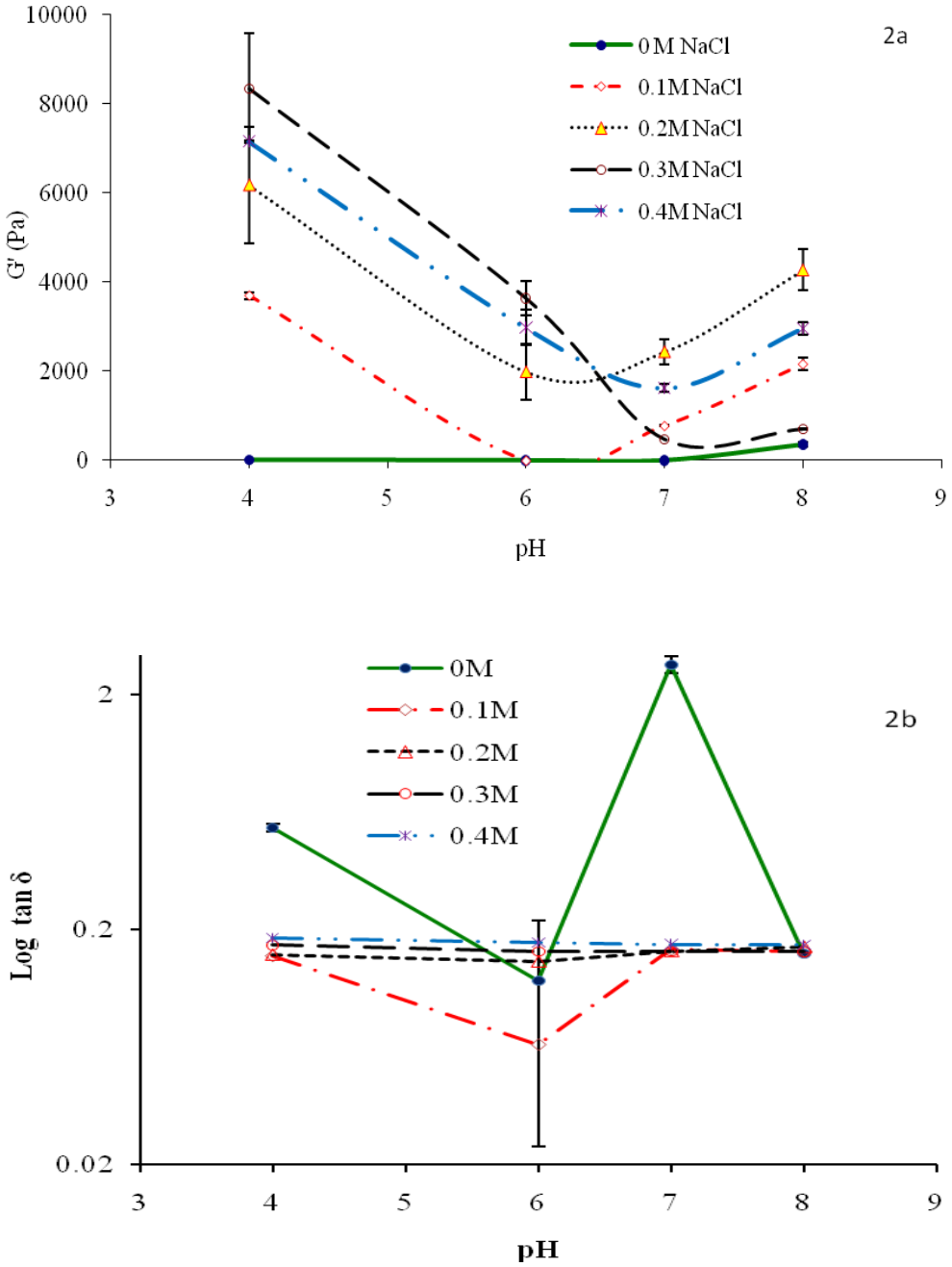

Fig. 4.2 Impact of combination of pH and NaCl on storage modulus G' and tan δ. PPIs concentration 14.5% (w/v). Error bars represent standard deviation. 2a: impact of combination of pH and NaCl on storage modulus G'; 2b: impact of combination of pH and NaCl on log tan δ.

The impact of the interaction between pH and NaCl concentration on rheological parameters focussed on lower salt concentrations to reflect the salt levels likely to be encountered in food systems. At pH values of 4 to 6, the presence of salt was required to get any structure formation and higher salt concentration up to 0.3 M resulted in higher G' values (Fig. 4.2). In this pH range the G' value for 0.4 M NaCl was not different from that for 0.3 M NaCl. The high G' values at pH 4 may simply be related to the lower denaturation temperatures at this pH, which are below the temperature used for gel formation. The finding of Renkema et al. (2000) who reported that higher G' values were achieved at pH 3.8 than at pH 7.6 when 10% soy protein isolate and glycinin dispersion in 0.2 M NaCl were heated to form gels is in agreement with ours. They attributed the difference in gel stiffness to the earlier onset of gelation at pH 3.8. Furthermore, they speculated that it was probably more important that different types of network were formed in terms of gel structure, which have a great impact on the rheological properties of the gels. They found that at pH 3.8, coarse gels with large aggregates, thick strands, and large pores were formed. The pH 7.6 gels consisted of much smaller aggregates and a more fine-stranded network structure with small pores. In coarse gels, more protein-protein interactions occurred between the particles. Furthermore, thick strands were more difficult to bend than thin ones. These both resulted in higher *G*' values. At pH 7 and 8, salt was again required to produce a protein network. Similar results were also obtained by van Kleef (1986) for SPI and glycinin and by Nagano et al. (1994) for β-conglycinin. Of the salt concentrations examined, 0.2 M NaCl produced the strongest gel followed by 0.4 M, 0.1 M and 0.3 M NaCl.

The lack of gel formation in the absence of salt was also apparent in the tan δ values which tended to be much higher than when salt was included. This was most noticeable at pH 7, where the high value and variability (2.68 ± 0.22) made data analysis for the samples which included salt difficult. The interactions between salt concentrations are shown in Fig. 4.2b, where the combination of low pH (4 or 6) and low salt concentration (0.1 or 0.2 M) produced a more elastic network than were obtained at higher pH values or higher salt concentrations.

While the stiffest gel was formed at pH 4 with 0.3 M NaCl, a low salt concentration produced a more elastic network leading to the conclusion that the strong network with 0.3 M NaCl may contain some aggregated material.

4.5. Conclusion

Pea protein gelation properties are influenced by both pH and salt. NaCl had a stabilization effect which, at higher concentration, inhibited pea protein from denaturation. The strongest gel stiffness was achieved at 0.3M NaCl at pH values below 6; at higher pH values lower salt concentrations produced stronger gels. The gelling temperature was also influenced by salt in that higher salt concentration resulted in higher gelling temperatures at pH 5.65. At a NaCl concentration of 2.0 M, pea protein gelation was completely suppressed. The influence of pH on gel formation was influenced by change in protein structure as evidenced by changes in thermal properties. Higher pH values in the 6 to 9 range resulted in higher denaturation temperatures and higher enthalpies of denaturation. As a result, gelling temperature was the highest at pH 6.0 (89.1 ºC). The protein unfolding at pH 4.0, as evidenced by the low T_d and ΔH values,

allowed for increased interaction between proteins and stiffer networks than at other pH values. Careful adjustment of pH and NaCl concentration are necessary to effectively utilize pea protein isolate as a functional additive.

Chapter 5: Gelation properties of salt-extracted pea protein isolate induced by heat treatment: Effect of heating and cooling rate (Sun, X. D. and Arntfield, S. D. 2010. *Food Chemistry*, 124, 1011–1016)

5.1. Abstract

Gel network formation of a salt extracted pea protein isolate was studied using dynamic rheological measurements. The gelling point was dependent on heating rate and was unaffected by cooling rate. When both the heating and cooling rates were increased (from 0.5 to 4 °C/min) final G' value decreased, indicative of decreased gel stiffness. During the heating phase, the storage modulus and loss modulus fluctuated below 1 Pa at almost constant values with the storage modulus smaller than the loss modulus until the gelling point was reached. The rate of cooling had a greater impact on the development of storage modulus than that of heating. Compared to the of commercial pea protein isolate (PPIc) and soy protein isolate (SPIc) at the same protein concentration, salt-extracted pea protein isolate (PPIs) was much stiffer than PPIc but weaker than SPIc. Careful control of the heating and cooling rates will enable maximum gel stiffness for heat-induced pea protein gel, thus enhancing utilization of pea protein as an additive in meat food industry.

5.2. Introduction

Although dry peas can be processed into products including pea flour, pea protein isolate (PPI), pea starch, and pea fiber, the application of pea protein in food products is limited because of its weak functionality as a food ingredient. Not many reports were found on gelation properties of PPI in the literature. Pea protein isolate is usually used as

a protein source in animal feed. If used as a substitute for meat protein or as a nutritious and functional additive in human foods, pea protein can play an important role similar to what is done with soy protein. However, pea protein isolate forms a weaker and less elastic gel than soy protein isolate when processed under the same conditions (Shand et al., 2007). Therefore, it is necessary to seek efficient methods to improve gelation properties of PPI through manipulation of processing conditions, which could enhance competitiveness of PPI as functional additive.

Globular proteins from various sources play important roles in many foods, both because of their nutritional value and their contribution to food texture (van Kleef, 1986). These textural contributions come from the network structures created by protein crosslinking. Since gelation is an important functional property of the globular proteins used to modify food texture (Ikeda & Nishinari, 2001), it is necessary to understand which factors determine the gel network formation and how they are affected by processing parameters. Such an understanding enables a better control of food texture and quality.

For globular proteins, protein-protein interactions usually occur following denaturation, often induced by heating. The heating temperature and heating rate are factors that have been shown to affect gel network formation by globular proteins (Matsumura & Mori, 1996). In fact, they are processing parameters that can be controlled to control gel formation.

Small strain oscillatory (dynamic) testing is useful in evaluating gelation properties and gel stiffness, because this method is extremely sensitive to changes in

physical structure and chemical composition of the sample (Westphalen et al., 2005). The method is suitable for following subtle changes associated with gel forming phenomenon (Hamann, 1987). High G' (storage modulus) values are indicative of stronger intermolecular network and increased interactions between proteins, while low tan δ values indicate a more elastic network (Uruakpa & Arntfield, 2006).

The gelling point determination from oscillatory measurements has been tested by several different methods, including the crossover of the storage (or elastic) modulus G' and the loss (or viscous) modulus G" (Clark, 1991; Friedrich & Heymann, 1988; Ikeda & Nishinari, 2001; Muller et al., 1991; Ross-Murphy, 1995; Winter, 1987; Yoon et al., 1999), linear extrapolation of the rapidly rising storage modulus G' to the intercept with the time axis (Hsieh et al., 1993; Steventon et al., 1991), and the maximum G" point (Stading & Hermansson, 1990). After the gel point, protein aggregates are bound together into a continuous molecular structure, as described by Hsieh & Regenstein (1992) and changes in these structures are reflected in the rheological data obtained.

In the study of O'Kane et al. (2004c), heating rate did not affect gel formation with pea legumin. However, it was observed that slower cooling of the legumin samples increased the gel stiffness. Studies on the effects of heating and cooling rate on the gelation properties of pea protein isolates extracted from different cultivars have also been reported (O'Kane et al., 2005). It was found that heating rate had no impact on gel formation of pea protein while slower cooling rates did influence the gel formation of all pea cultivars and, in general, increased the gel stiffness.

Although some studies have been done to investigate the influence of heating and cooling rates on the gelation properties of heat-induced pea protein isolate, none of these studies investigated gelation properties of pea protein isolate when the protein was extracted by salt and precipitated by dilution.

The objective of this study was to investigate the impact of heating and cooling rates on the gelation properties of salt extracted pea protein isolate induced by heat treatment and to determine the optimal heating and cooling rate to maximize . This will provide valuable processing parameters for utilization of this protein in products such as meat.

5.3. Materials and methods

5.3.1. Commercial pea flour, pea protein isolate, soy protein isolate and PPIs extraction procedure

Commercial pea flour and commercial pea protein isolate (PPIc) were kindly donated by Nutri-Pea Ltd. (Portage la Prairie, MB, Canada). The Century Flour was made from Canadian yellow pea by milling following tempering; protein content was greater than 25%. PPIc—Propulse Pea Protein contained 82% protein. The salt extracted pea protein isolate (PPIs) was prepared from the Century Flour using a salt extraction method as described previously (Sun & Arntfield, 2010). Sodium chloride (0.3 M) was employed to extract pea protein from pea flour (pea flour:sodium chloride solution = 3:10, w/v), the soluble pea proteins were then separated by centrifugation (2795×g, 20 min) and precipitated by dilution in cold distilled water (supernatant:water = 1:2, v/v). The protein was resuspended in distilled water and unwanted salt was removed from pea

protein concentrate by dialysis. A molecular porous membrane tubing (*Spectra/Por*©) with molecular weight cut-off of 12–14,000 Da was used. The desalted protein isolate was then freeze dried (Genesis SQ Freeze Dryer, Gardiner, NY, USA). Through this extraction procedure, a pea protein isolate with low denaturation was obtained. Following freeze drying, the PPIs contained 81.9% of protein as determined by Kjeldahl method using an N to protein conversion factor of 5.7 (AACC, 1982). Commercial soy protein isolate (SPIc) (PRO-FAM 974) was obtained from Archer Daniels Midland Company (ADM) (Decatur, IL, USA) and protein content was reported to be 90%.

5.3.2. Differential Scanning calorimetry (DSC)

The thermal properties of the salt-extracted pea protein isolate suspensions were examined using a DSC Q200 (TA Instruments, New Castle, DE, USA). Instrumental conditions were as described in Shand et al. (2007). Peak transition temperature or denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed from the endothermic peaks observed in the thermograms. In a typical experiment, 60–70 μL of the 10.5% protein sample was sealed in a preweighed stainless steel high volume pan and weighed again to determine sample weight. An empty pan was used as reference. The sample was heated over a temperature range of 30–140 ºC in a standard DSC cell that had been calibrated with both indium and sapphire standards. Thermal curves were obtained at a heating rate of 0.5, 1.0, 2 and 4 ºC/min. ΔH values were reported in J/g protein. Each sample was analysed in duplicate.

5.3.3 Rheology

The pea protein isolate was mixed with 0.3 M NaCl (Fisher Scientific, Ottawa, Canada) to obtain a suspension of protein isolate at a concentration 14.5% (w/v). Good gel formation has been reported for the pea protein at this salt and protein concentration compared to other salt and protein concentrations (Sun & Arntfield, 2010). To achieve complete suspension, the samples were mixed by a Vortex-Genie Mixer (Scientific Industries Inc., Bohemia, NY, USA) for 1 min. The sample was then loaded into a TA2000 rheometer (TA Instruments, Newcastle, Del. USA). The pH of the suspension was between 5.65 and 5.70. For each sample, approximately 1 mL of the pea protein isolate suspension was transferred to the lower plate of the parallel plate geometry of the rheometer. The upper plate was lowered to give a gap width of 1.00 mm. A thin layer of light mineral oil was added to the well of the upper plate geometry and a solvent trap cover was used to prevent sample drying during heating. In this way, a water-saturated atmosphere was maintained at the surface of the sample. The following heating protocol was used. Samples were first equilibrated at 25 ºC for 2 min, then heated and cooled over a temperature range of 25–95–25 ºC at a controlled rate (4 ºC/min, 2 ºC/min, 1 ºC/min, or 0.5 ºC/min; these heating and cooling rates were practical and often used by other researchers). The temperature range was from room temperature to 95 ºC, because plant proteins have high denaturation temperatures, and 95 ºC was sufficient to denature pea proteins and is practical for the food industry. Rheological data were collected for every degree change during heating and cooling. This was followed by a frequency sweep over a range of 0.01–10 Hz at 25 ºC. Through this procedure, both changes during gel preparation and characteristics of the final gel were collected. The storage modulus (G') and loss modulus (G") were determined as a function of frequency for each sample. The

loss tangent or tan delta (tan δ = G"/G'), a measure of the energy lost due to viscous flow compared to the energy stored due to elastic deformation in a single deformation cycle, was also calculated. The input amplitude strain used for the dynamic analysis was 0.02, a value found to be in the linear viscoelastic region for heat-induced protein networks in preliminary experimentation. Samples were run in duplicate. The gelling point temperatures for the pea protein suspensions at different heating and cooling rates were determined by extrapolating the rapidly rising storage modulus G' during the initial heating phase to intercept the temperature axis.

5.3.4. Statistical analysis

All data were analysed by one way analysis of variance (ANOVA) for significant differences, with minimum significance set at the 5% level ($P < 0.05$), followed by Tukey's test to find differences. GraphPad InStat software version 3.06 (GraphPad Software Inc. La Jolla, CA, USA) was used for the statistical analysis.

5.4. Results and discussion

For all heating and cooling rates, the curves obtained during the heating phase were essentially the same, with both G' and G" below 1 Pa, and G' lower than G", until the gel point was reached. As a result, comparison of heating and cooling rates has been done by comparing changes during the cooling phase (Figs. 5.1, 5.2).

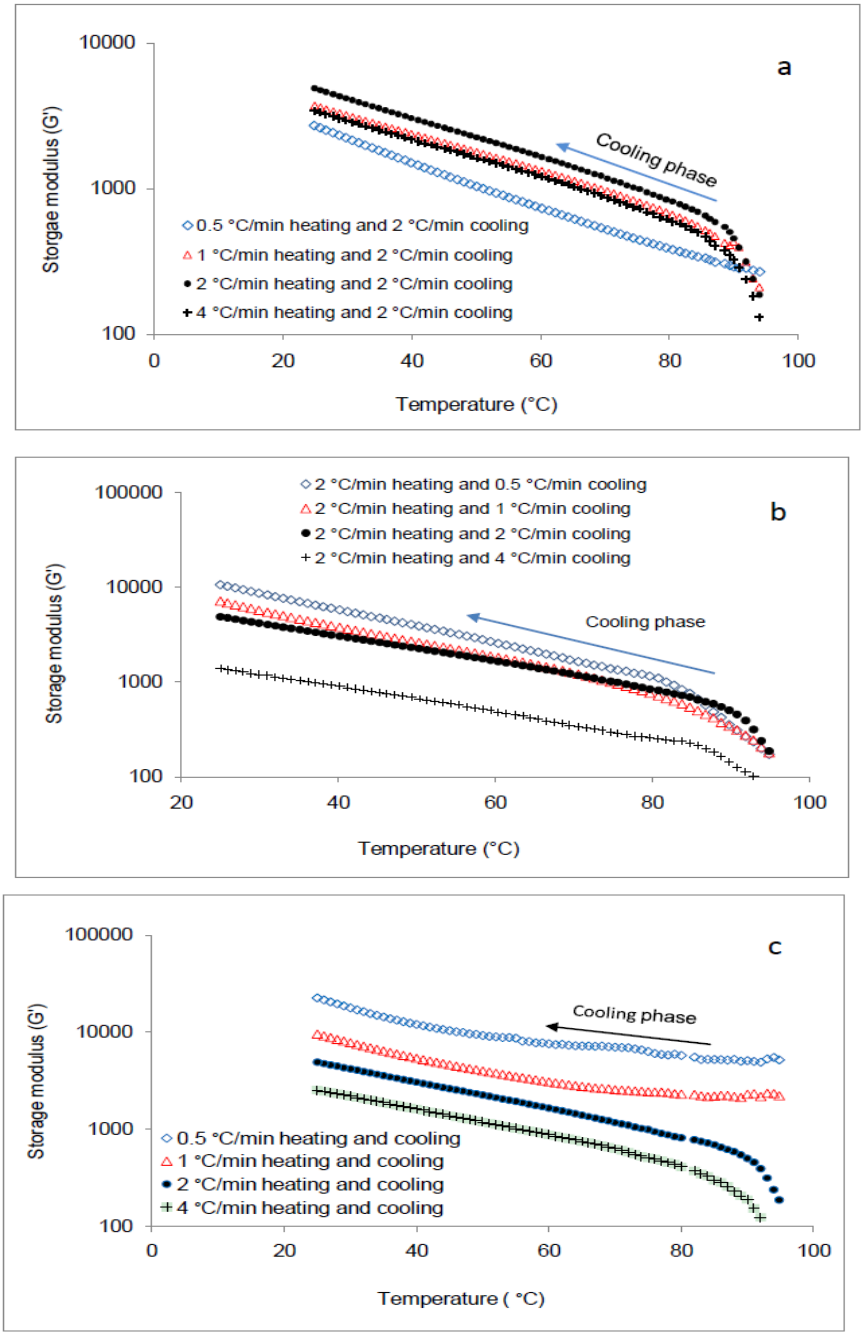

Fig. 5.1 Effect of different cooling and heating rates on the development of the storage modulus (G') during cooling of a salt extracted pea protein isolate at a concentration of 14.5% (w/v) in 0.3 M NaCl: (a) effect of heating rate at cooling rate of 2 ºC/min; (b) effect of cooling rate at heating rate of 2 ºC/min; (c) effect of varying both heating and cooling rate.

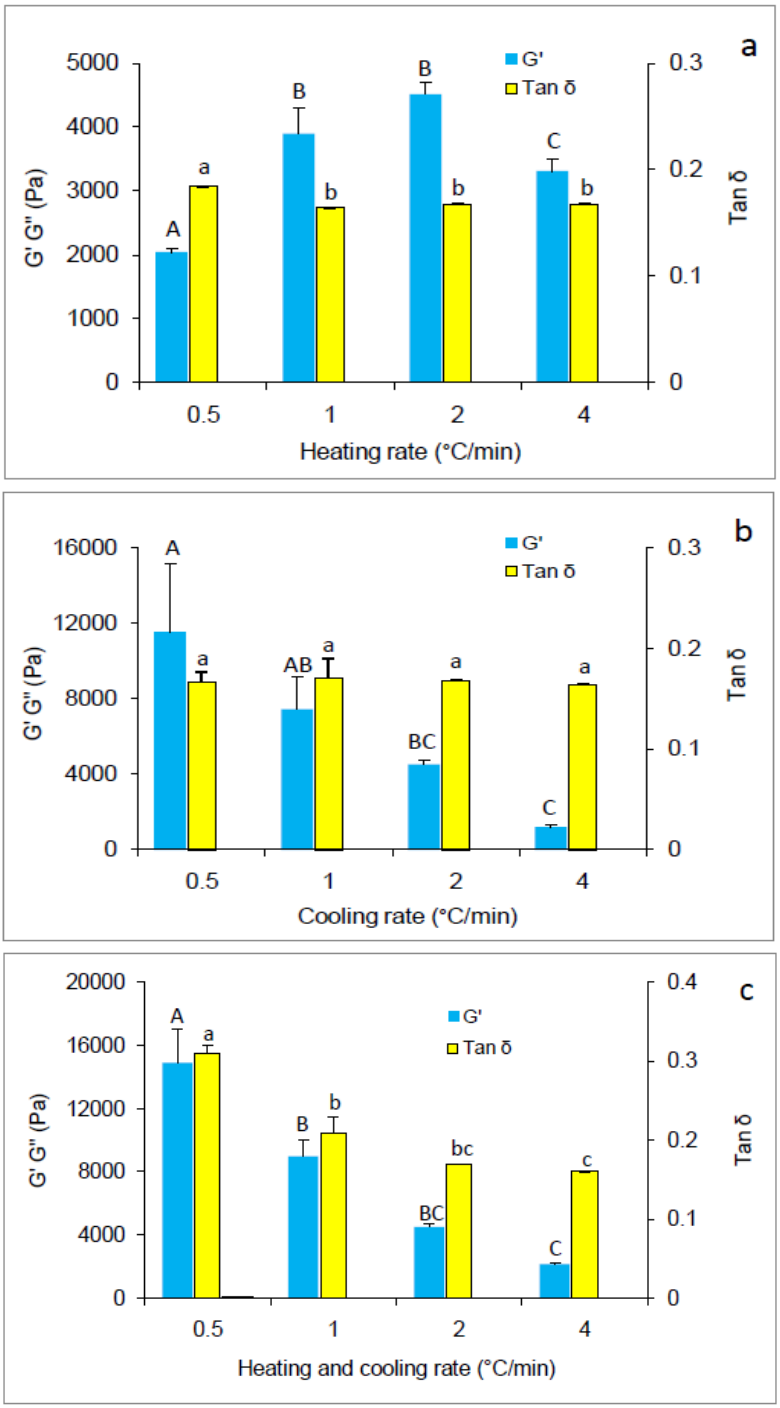

Fig. 5.2 Gel properties of heat-induced pea protein isolate at 1 Hz from the sweep frequency of gels formed at concentration of 14.5% (w/v) in 0.3 M NaCl: (a) effect of heating rates, (b) effect of cooling rates, (c) effect of heating and cooling rate.

5.4.1. Impact of heating rate on the development of storage modulus during the cooling phase of gel formation

When heated at the slowest heating rate of 0.5 °C/min, the values for the storage modulus during the cooling phase were lower than those measured at the faster rates of 1°C, 2 °C/min and 4 °C/min (Fig. 5.1a). In addition, it can be seen that using a heating rate of 2 °C/min, the G' values observed during the cooling phase were the greatest. It was expected that at the same cooling rate, slower heating rates would enable pea protein molecules to have more time to rearrange and align into a more ordered network structure with increased gel stiffness. However, this was not the case, except that the G' values for the highest heating rate (4 °C/min) were lower than at 2 °C/min. The results of O'Kane et al. (2004c) did not support this theory either. They found that at the same cooling rate (1 °C/min), heating rate did not affect the gel formation by pea legumin. Surprisingly, in this study a slower heating rate seemed to have some detrimental effect on the development of G' during gel formation. There appeared to be an optimal heating rate (2 °C/min) to maximize the rearrangement and alignment of proteins to produce the greatest storage modulus.

Fig. 5.2a shows the properties of heat-induced pea protein gels formed at different heating rates and at 2 °C/min cooling rate. The G' value is significantly lower and the tan δ value significantly higher for pea protein heated at 0.5 °C/min than those obtained at the higher heating rates. However, the tan δ values were not significantly different at heating rates greater than 0.5 °C/min. The G' values for the samples heated at 1 °C/min and 2 °C/min achieved the highest values and were not significantly different, but were also significantly greater than the sample heated at 4 °C/min. This is similar with the

result of Renkema & van Vliet (2002), who observed that soy protein gels prepared at a heating rate of 5 K/min had a lower G' value than gels heated at 1 K/min. Arntfield & Murray (1992), Stading et al. (1993) and Li et al. (2006) have also observed the same phenomenon for other globular proteins such as vicilin, ovalbumin, β-lactoglobulin and whey protein. Renkema & van Vliet (2002) explained the aggregation kinetics might have been affected by the higher heating rate thus resulting in gels with a different network structure (different strand thickness, pore size, or curvature). This point of view was supported by the findings of Mellema et al. (2002) and Renkema (2001), which indicated that differences in network structure affect G' value. It is not clear why gels prepared at the slowest heating rate of 0.5 °C/min had lower G' value than gels heated at higher rates. One possible reason is that proteases activity breaks down the formed gel network. This may also be associated with aggregation kinetics, but requires further investigation. The maximum gel stiffness and relative elasticity of the gel are achieved at 2 °C/min heating rate; this seems to be the optimum heating condition for pea protein isolate.

5.4.2. Impact of cooling rate on the development of storage modulus during the cooling phase of gel formation

Cooling rates influence the gel formation of pea protein isolates (Fig. 5.1b). When using the same heating rate, the slowest cooling rate (0.5 °C/min) enabled pea protein isolate to form the stiffest gel among the four treatments. This is consistent with the result of O'Kane et al. (2004c) and O'Kane et al. (2005) who showed increased gel stiffness with slower cooling rate. They suggested that slow cooling could maintain the protein in its unfolded state for a longer time, slowing down the reactivity of the exposed residues,

and enabling more optimal arrangement to occur, prior to interactions between proteins. Since hydrogen bonds are favored at low temperature and it has been confirmed that one of the main forces involved in promoting gel structure of pea legumin protein is the hydrogen bonds (O'Kane et al., 2004c). It can be speculated that the hydrogen bond formation is enhanced at slower cooling rate resulting in stronger gels. O'Kane et al. (2004c) also indicated that under a slower cooling rate (1 °C/min heating and 0.2 °C/min cooling), hydrophobic interaction and disulfide bond are involved in formation of gel structure of pea legumin protein. They explained that disulfide bonds became involved in the gel network since a slow cooling rate provided time for a reaction between sulfhydryl group and as a result, gel stiffness was enforced. It is well known that hydrophilic amino acids such as asparagine, glutamine, serine and threonine have polar groups on their side-chains and are capable of forming hydrogen bonds which can provide links between unfolded proteins. For hydrophobic interaction, non-polar amino acids such as alanine, glycine, leucine, isoleucine, valine and phenylalanine are more likely to be involved and are exposed from the globular protein interior during protein denaturation.

At a 2 °C/min heating rate, cooling rates had an impact on the properties of heat-induced pea protein gels (Fig. 5.2b). The effect of cooling rate was in contrast to what was seen for heating rate and G' decreased with higher heating rates. However, tan δ values for the four treatments were not significantly different. Similar results were obtained by Renkema & van Vliet (2002), who speculated that during cooling phase, the increase in G' was most likely caused by a decreasing mobility of soy proteins with declining temperature, thus bond formation within and between the protein molecules was enhanced. Therefore, it can be extrapolated that while the relative amount of

crosslinking within the gel does not change with cooling rate, the overall relatively lower gel stiffness at higher cooling rates is caused by less bond formation within and between pea protein molecules.

5.4.3. Impact of changes in both heating and cooling rates on the development of storage modulus during the cooling of gel formation

Changing both heating and cooling rates also has a significant impact on G' of a pea protein isolate when samples are heated and cooled at the same rate. As expected, slower heating and cooling rates induced greater gel stiffness than faster heating and cooling during cooling of pea protein (Fig. 5.1c). This is consistent with the result of O'Kane et al. (2005), which also indicated that slower heating and cooling rate led to greater G' value than faster one. It is noteworthy that the shape of the curves for the slower heating and cooling rates (0.5 °C/min and 1 °C/min) are different from the curves for faster heating and cooling (2 °C/min and 4 °C/min). At the slower heating and cooling rates, G' is high at the beginning of the cooling phase, then gradually increases with a higher rate of change at temperatures below 50 °C. With the higher heating rates, a sharp increase in G' was noted at the onset of cooling, but once the temperature was below ~90 °C, there was a constant increase in G' with a decrease in temperature. At the faster heating and cooling rates, it would appear that the network was still forming during the cooling phase while for the slower heating rates the networks formed during heating were strengthening during the cooling phase.

Changing both heating/cooling rates also had an impact on the properties of heat-induced pea protein network (Fig. 5.2c). Both G' and tan δ decreased in response to an

increase in the heating and cooling rate. For G', the gradual decrease with increasing heating and cooling rates is similar to what was seen when cooling rate was increased at a constant heating rate (Fig. 5.2b) and provides further evidence that a slower rate of cooling leads to improved interaction between proteins and a stronger network. However, the type of network, as reflected by the relative contribution of protein-protein interactions to the elastic and viscous components of the network (measured by tan δ) was primarily influenced by the heating rate. When only cooling rate was changed, tan δ was unaffected (Fig. 5.2b), but when only heating rate was changed (Fig. 5.2a), a less elastic network with higher tan δ values was obtained with the 0.5 °C/min heating rate. A similar effect was seen when both heating and cooling rate were slower.

5.4.4. Effect of heating and cooling rates on gelling point

For the impact of heating rate (with identical cooling rate, 2 °C/min) on gelling point, an increase in heating rate resulted in an increased gelling point, regardless of cooling rate (Table 5.1). This is consistent with the observation of O'Kane et al. (2004c); who showed a slower heating rate initiated gel formation at a lower temperature. This is also the case for soy protein gelation as reported by Renkema & Van Vliet (2002), who indicated that at faster heating rates, denaturation temperatures are higher, which resulted in the higher gelation temperature (gelling point) than slower heating rates. Similar phenomena have also been reported for egg white (Donovan et al., 1975), vicilin, and ovalbumin (Arntfield & Murray, 1992). Therefore, it can be concluded that the gelling point was heating rate dependent. At a lower heating rate the protein molecules have more time to rearrange and align, thus they begin to crosslink at lower temperature,

whereas at a higher heating rate, protein molecules do not have sufficient time to rearrange and align, thus they start to crosslink later.

As expected, cooling had no influence on gelling point, as indicated by the similar gelling points obtained at the same heating rate and different cooling rates (Table 5.1). When both heating and cooling rates were varied, it was only the heating rate that influenced the gelling point.

Table 5.1 Effect of different heating and cooling rate on gelling point

Heating rate * (°C/min)	Cooling rate * (°C/min)			
	0.5	1	2	4
0.5	84.1 ± 0.1 [a]	___	83.6 ± 0.5 [a]	—
1	—	86.6 ± 0.2 [b]	86.8 ± 0.3 [b]	—
2	88.5 ± 0.6 [c]	88.9 ± 0.6 [c]	88.9 ± 0.2 [c]	88.7 ± 0.5 [c]
4	___	___	91.5 ± 0.5 [d]	91.5 ± 0.4 [d]

[*] Means ± SD of duplicates.

[a~d] Column or row values followed by the same superscript letter are not significantly different (P<0.05).

Gelling points of all four treatments were higher than those of the DSC onset gelation temperature (T_m) and lower than those of the corresponding DSC denaturation temperature (T_d) (Table 5.2). This is in agreement with the results of Meng & Ma (2002) and Zheng et al. (1993b). They found that no gel was formed below T_m and G' value

gradually increased until 95 ºC, higher than the T_d of red bean globulin and broad bean legumin. They also reported that at different heating rates, the tendency was for gelling points (gelation temperature) to increase with increased heating rate. They observed significant differences and also reported that T_m, T_d, and ΔH tended to increase with increased heating rate, but no significant differences were observed for T_m and ΔH. Therefore, it can be concluded that less energy is needed and decreased DSC onset gelation temperature and denaturation temperature are associated with denaturation of pea protein prior to gel formation at slower heating rates.

Table 5.2 Effect of heating rate on differential scanning calorimetry (DSC) transition temperatures of pea protein isolate

Heating rate	T_m *	T_d *	ΔH *
0.5	70.8±0.8^{a}	90.5±0.0^{a}	8.3±2.5^{a}
1.0	72.2±0.8^{a}	91.5±0.4^{ab}	14.3±2.8^{a}
2.0	71.6±0.1^{a}	92.3±0.1^{bc}	16.8±0.5^{a}
4.0	72.7±0.5^{a}	93.3±0.4^{c}	16.5±2.1^{a}

T_m: DSC onset denaturation temperature.

T_d: DSC denaturation temperature.

ΔH: Protein denaturation enthalpy.

* Means ± SD of duplicates.

$^{a\sim c}$ Column values followed by the same superscript letter are not significantly different (P<0.05).

5.4.5. Comparison of gel stiffness of PPIc, PPIs and SPIc

Since SPIc is the most commonly used vegetable protein in meat products, comparison of the gelation properties of PPIc, PPIs and SPIc is essential for evaluating the usefulness of PPIs as a functional additive in meat products. Maximum gel stiffness was obtained for SPIc, followed by PPIs, while PPIc formed the weakest gel (Table 5.3). This is in agreement with Shand et al. (2007) who also indicated that pea protein isolate could only form a weaker and less elastic gel than soy protein isolate under similar processing conditions. O'Kane et al. (2004c) observed that it was mainly hydrogen bonds and hydrophobic interactions which supported network formation of legumin proteins (11S protein) in pea and soy, whereas disulfide bonds had minimum involvement. O'Kane et al. (2004c) and Soral-Śmietana et al. (1998) also found that soybean glycinin (11S protein) was able to form a better network than pea legumin (possibly due to the availability of lysl and glutaminyl residues) based on the textural properties of the heat-induced gels. However, at 2 ºC/min heating and cooling rate, the gel stiffness of PPIs was greatly increased compared to PPIc.

Table 5.3 Comparison of gelation properties of PPIc, PPIs and SPIc. Protein concentration of all samples were 10.5% (w/v), dispersed in 0.3M NaCl. Heating and cooling at 2 ºC/min.

	PPIc	PPIs	SPIc
G', Pa *	1.97±0.7[a]	291.6±4.2 [b]	890.1±41.4 [c]
G", Pa *	2.55±3.1 [a]	48.8±1.4 [b]	111±9.1 [c]
Tan delta *	1.751±0.70 [a]	0.167±0.00 [a]	0.124±0.00 [a]

* Means±SD of duplicates.

[a~c] Row values followed by the same superscript letter are not significantly different ($P<0.05$).

5.5. Conclusion

Heating and cooling rates influence the gelation properties of heat-induced pea protein and the properties of the resulting gels. With increasing heating rate (from 0.5 to 4 °C/min), at the same cooling rate (2 °C/min), the gelling point tended to increase whereas the storage moduli (G') were not statistically changed. With increasing cooling rate (from 0.5 to 4 °C/min), at the same heating rate (2 °C/min), the storage moduli decreased whereas gelling point remained constant. The storage moduli (G') were not statistically influenced by heating rate but were affected by cooling rate. The G' values in the resulting gels increased with decreasing cooling rates. The effect of heating rate was seen in tan δ values; a lower tan δ value, indicative of a more elastic network, was obtained with higher heating rates, regardless of cooling rate. The gelling point was also influenced by heating rates only and tended to increase with increasing heating rates. Cooling rates had no impact on the gelling point. The utilization of pea protein as a food ingredient can be achieved by careful control of the heating and cooling rates during processing, to produce the appropriate gel characteristics. This will enhance the utilization of pea protein isolate as a functional additive by improving product elasticity with potential application in meat products.

Chapter 6: Molecular forces involved in heat-induced pea protein gelation: effects of various reagents on the rheological properties of salt-extracted pea protein isolate (Submitted to *Food hydrocolloids* on Oct. 1, 2010)

6.1. Abstract

The molecular forces involved in the gelation of heat-induced pea protein gel were studied by monitoring changes in gelation properties in the presence of different chemicals. At 0.3 M concentration, sodium thiocyanate (NaSCN) and sodium chloride (NaCl) showed more chaotropic characteristic and enhanced the gel stiffness, whereas sodium sulfate (Na_2SO_4) and sodium acetate (CH_3COONa) stabilized protein structure as noted by increasing denaturation temperatures (T_d) resulting in reduced storage moduli (G'). To determine the involvement of non-covalent bonds in pea protein gelation, guanidine hydrochloride (GuHCl), propylene glycol (PG), and urea were employed. The significant decrease in G' of pea protein gels with addition of 3 M GuHCl and 5 M urea indicated that hydrophobic interaction and hydrogen bonds are involved in pea protein gel formation. The increase in G' with increasing PG concentration (5-20%), demonstrated hydrogen bonds and electrostatic interaction involvement. No significant influence was observed on G' with addition of different concentrations of 2-ME, DTT, and up to 25 mM NEM, which indicated that disulfide bonds do not contribute much to pea protein gel stiffness. Reheating and recooling demonstrated that gel formation during the initial cooling phase was thermally reversible but not all the hydrogen bonds disrupted in the reheating stage were recovered when recooled.

6.2. Introduction

The gel forming ability of pea proteins upon heating is an important functional property, which affects their utilization in foods. To further the adoption of pea proteins as functional additives in foods, it is essential to understand their gelation mechanism. Heat-induced gels have been prepared from pea protein isolates (Shand et al., 2007) and pea protein isolates containing transglutaminase (Shand et al., 2008). In these applications, the pea protein isolates were extracted by pH adjustment and were recovered by isoelectric precipitation. The contributions of constituent proteins to gel formation and the molecular forces involved in gelation have been studied for protein isolates (Bora et al., 1994), purified vicilin (O'Kane et al., 2004a) and purified legumin (O'Kane et al., 2004b; O'Kane et al., 2005) again using materials recovered by isoelectric precipitation. From these studies, information is available on the gelation of legumin, vicilin and pea protein isolates that have been recovered by pH manipulation and isoelectric precipitation.

Protein gelation is the cross-linking of its polypeptide chains to from a three-dimentional network. Cross linking of proteins is caused by different molecular forces and may involve hydrogen bonds, ionic attractions, disulphide bonds, hydrophobic associations or a combination of the above (Otte et al., 1999). A range of techniques have been used to investigate molecular forces (Table 6.1).

The molecular forces involved in the gel network are dependent upon the protein, and protein structure which can be influenced by the method used for protein isolation (Utsumi & Kinsella, 1985a; Shimada & Matsushita, 1980). The involvement of different

interaction forces in formation and structure of protein gels can be deduced from effects of pH, salts, reducing agents and dissociating agents (Clark et al., 1981; Utsumi & Kinsella, 1985a; Mulvihill et al., 1990). To determine which molecular forces are involved in formation of heat-induced pea protein gel, different chemical reagents can be employed.

Hydrogen bonds and hydrophobic interactions in protein can be destabilized by urea and guanidine hydrochloride (GuHCl). Urea is usually used to denature proteins, but the mechanism is not completely understood. Urea denatures a protein molecule through preferential adsorption with charged protein solutes, dehydrating the molecules and causing repulsion between proteins, stabilizing the unfolded form (Wallqvist et al., 1998). Consequently, urea probably interferes with both hydrophobic interactions and hydrogen bonding by dehydrating protein molecules and interacting through hydrogen bonds that might otherwise interact with the solvent surrounding the molecule.

Sulfhydryl/disulfide interchange has been proposed to be involved in soy protein gelation based on the reaction of the gel (a loss of the gel integrity) to several reagents: β-mercaptoethanol (β-ME or 2-ME) (Briggs & Wolf, 1957; Wolf & Briggs, 1958; Castimpoolas & Meyer, 1970; Utsumi et al., 1984; Utsumi & Kinsella, 1985b; Wolf, 1993), dithiothreitol (DTT) (Utsumi & Kinsella, 1985a; Wolf, 1993; McKlem, 2002), and N-ethylmaleimide (NEM) (Briggs & Wolf, 1957; Wolf & Briggs, 1958; Castimpoolas & Meyer, 1970; Utsumi & Kinsella, 1985a; Shimada & Cheftel, 1988; Wang & Damodaran, 1990). When electrostatic forces are involved in gel formation of whole plasma protein, gel strength is affected by pH and salts (Hickson et al., 1980; Hermansson, 1982a, b;

O'Riordan et al., 1988a, b). Therefore, involvement of electrostatic interactions can be determined by the effect of salts and pH. The effects of various reagents are summarized in Table 6.1.

Table 6.1 Effect of various reagents on molecular forces exist in protein

	Non-covalent bonds			Covalent bond	References
	Electrostatic interaction	Hydrophobic interaction	Hydrogen bond	Disulfide bond	
DTT				Disrupt	Rüegg & Rudinger (1977)
GuHCl		Disrupt	Disrupt		Tanford (1968)
2-ME				Disrupt	
NEM				Disrupt	Creighton (1993)
PG	Promote	Disrupt	Promote		Tanford (1962)
Urea		Disrupt	Disrupt		Gordon & Jencks (1963)

More experimental data are needed to better understand the importance of various forces in network formation and to ascertain their impact on rheological properties of pea protein gels, particularly gels formed by pea proteins that have been isolated by salt extraction and precipitation by dilution. As no pH adjustments are used in this isolation method there should be no changes in protein structure due to pH manipulation. Involvement of hydrophobic interactions and hydrogen bonds during gelation of isoelectrically precipitated pea protein have been reported previously (O'Kane et al., 2004b), but no work has been done using various salts to investigate the role of electrostatic interactions. The objective of our current research was to evaluate the effects

of various salts (NaSCN, Na_2SO_4, CH_3COONa, NaCl), chemicals that target non-covalent interactions (propylene glycol [PG], DTT, urea, GuHCl), chemicals that target covalent interactions (NEM, DTT and 2-ME) as well as reheating and recooling on the properties of pea protein gels prepared from a salt extracted pea protein isolate. This allowed us to elucidate the molecular forces involved in gel network formation and maintenance.

6.3. Materials and methods

6.3.1. Commercial pea flour, PPIc, and PPIs

Commercial pea flour and pea protein isolate were kindly donated by Nutri-Pea Ltd (Portage la Prairie, MB, Canada). The flour was made by milling dehulled Canadian yellow pea, and its protein content was greater than 25%. The pea protein isolate (PPI) was prepared by a salt-extraction method described previously (Sun & Arntfield, 2010). After freeze drying (Genesis SQ Freeze Dryer, Gardiner, NY, U.S.A.), the PPI contained 81.9% protein as determined by Kjeldahl method using an N to protein conversion factor of 5.7 (AACC, 1982).

6.3.2. Rheology

All samples were prepared with 14.5% pea protein isolate at its natural pH. Control was prepared with distilled water without any salt. For the salt series, 0.3 M solutions of Na_2SO_4 (Fisher Scientific, Fair Lawn, New Jersey, USA), CH_3COONa (Mallinckrodt, Inc., Pointe-Claire, Quebec, Canada), NaCl (Fisher Scientific, Ottawa, Canada) and NaSCN (Fisher Scientific, Fair Lawn, New Jersey, USA) were prepared with distilled water. GuHCl (electrophoresis grade; Fisher Scientific, Fair Lawn, New

Jersey, USA), urea (Fisher Scientific, Nepean, Ontario, Canada), DTT (Sigma Chemical Company, St. Louis, USA), NEM (Sigma Chemical Company, St. Louis, USA), PG (Sigma-aldrich, Inc., St. Louis, USA), NaSCN and 2-ME (MP Biochemicals Inc., Solon, Ohio, USA) were dissolved in 0.3M NaCl solution to produce the desired concentrations. To achieve complete suspension, samples were mixed by a Vortex-Genie Mixer (Scientific Industries Inc., Bohemia, N.Y., USA) for 1 min prior to loading to a TA 2000 rheometer (TA Instruments, Newcastle, Del. USA).

Rheological parameters were determined using the method of Sun & Arntfield (2010).

6.3.3. Differential Scanning calorimetry (DSC)

The thermal properties of salt extracted pea protein isolate and commercial pea protein isolate suspensions were examined using a DSC Q2000 and high volume pans. Instrumental conditions were as described in Shand et al. (2007). Peak transition temperature or denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed from the endothermic peaks observed in the thermograms using computer software (Universal Analysis 2000, Version 4.5A, TA Instruments, New Castle, DE). Thermal curves were obtained using 10-20 µL of sample at a concentration of 10% and a heating rate of 10 ºC/min with an empty pan as reference. The sample was heated over a temperature range of 30-120 ºC in a standard DSC cell that had been calibrated with both indium and sapphire standards. Each sample was analyzed in duplicate.

6.3.4. Statistical analysis

All data were analyzed for significant differences, with minimum significance set at the 5% level ($P<0.05$), using Tukey's test by GraphPad InStat software version 3.06 (GraphPad Software Inc. La Jolla, CA, USA).

6.4. Results and discussion

6.4.1. Effect of sodium salts

The thermal denaturation data for the pea protein isolate in the 0.3 M salts indicated that the denaturation temperature increased when salts were present with the greatest increase for Na_2SO_4, followed by CH_3COONa, NaCl and finally NaSCN (Table 6.2). The effects of salts on protein structure involve three mechanisms: electrostatic shielding effects, non-specific charge neutralization and direct ion-macromolecule interactions (Zhang & Cremer, 2006). It is believed that the ion specific effects arise from changes in the hydrophobic core of the protein (Zhang & Cremer, 2006). At low ionic strengths, salts are believed to primarily influence electrostatic interactions by interacting with charged groups on the proteins, whereas at higher concentrations the ion specific effects, or lyotropic effects, become prominent (Damodaran & Kinsella, 1981). At these higher concentrations, NaSCN has been shown to be a destabilizing salt while Na_2SO_4 stabilizes protein structure (von Hippel & Schleich, 1969; Damodaran & Kinsella, 1981). The data in this study support the possibility of a lyotropic effect at 0.3 M salt as the order of increase in denaturation temperature corresponded to the position of these salts in the lyotropic series. Melander & Horváth (1977) indicated that the property of a salt that affected hydrophobic interactions in proteins was determined by its molal surface

tension increment (σ) independently of the salt concentration. They also pointed out that σ formed the basis of a natural lyotropic series. Within this series, NaSCN is considered to be a destabilizing salt, with a strong ability to bind to proteins. Not only does this change the protein charge, but by creating an excessive negative charge, the SCN^- facilitates unfolding of the globular proteins at lower temperatures, thus explaining the low denaturation temperature observed with this salt. In contract, SO_4^{2-} anions are considered to be stabilizers of protein structure. This is reflected in the increased denaturation temperature. It would appear that non-polar groups are further buried within the protein structure resulting in higher temperatures to promote unfolding.

Although the inclusion of salts increased the thermal denaturation temperatures, with the exception of NaCl, the ΔH values for the pea protein in the 0.3 M salts were lower than that of the 10% pea protein dispersed in water (no salt) at its natural pH (5.65). For the NaSCN, changes in protein conformation due to the increased net charge would explain the lower ΔH value. Conformational changes due to stabilization may also account for the lower ΔH values for the Na_2SO_4 and CH_3COONa. Damodaran & Kinsella (1981) indicated that although higher denaturation temperature values reflect resistance to thermal denaturation, the tertiary and quaternary structures of the stabilized protein might not be the same as that of the native protein, and a lower ΔH value can result.

In the presence of the 0.3 M Na_2SO_4, CH_3COONa, NaCl, and NaSCN, rheological data for the pea protein isolate showed improved structure development for all four treatments in comparison to the water control (Table 6.2). Poor solubility for the water sample may account for the lack of network formation. Among the four treatments,

the SCN^- anion showed the greatest increase in G' values followed by Cl^-, CH_3COO^-, and SO_4^{2-}. It appears that the ability of the pea protein to form gels in the presence of 0.3 M sodium salts was, in part, related to the thermal denaturation temperature and therefore also dependent upon the lyotropic influence of these salts. For CH_3COO^-, and SO_4^{2-}, the denaturation temperature was increased to values in excess of 95 ºC, the temperature used for gel formation. As a result, the degree of protein unfolding was limited, thus reducing the potential for network formation. The high G' value with SCN^- resulted from the lower denaturation temperature and exposure of reactive groups as well as electrostatic shielding effects which minimized charge repulsion within the protein. While these salts affected gel stiffness differently they did not impact the relative elasticity of the networks formed as the tan δ was not significantly affected by the salt used (Table 6.2).

Table 6.2 Effect of 0.3 M sodium salt on the thermal denaturation of 10% PPIs at natural pH (5.65) and rheological properties of 14.5% PPIs gels (pH 5.65) at 1 Hz sweep frequency.

Salt	T_d*	ΔH*	G' *	Tan δ*
Control	86.2±0.1 [a]	15.8±0.0 [c]	0.35±0.2 [a]	1.36±0.6 [b]
Sodium sulfate (Na_2SO_4)	104.6±0.0 [d]	11.6±0.9 [ab]	248.5±62.9 [a]	0.18±0.0 [a]
Sodium acetate (CH_3COONa)	98.0±0.1 [c]	13.5±0.4 [b]	1198±196.6 [a]	0.17±0.0 [a]
Sodium chloride (NaCl)	94.3±0.3 [b]	17.8±0.2 [c]	4516±188.1 [b]	0.17±0.0 [a]
Sodium thiocyanate (NaSCN)	93.6±0.0 [b]	10.7±0.3 [a]	14560±1866.8 [c]	0.16±0.0 [a]

* Mean±SD of duplicate.

* Column values followed by the same superscript letter are not significantly different ($p<0.05$).

As NaSCN is a chaotropic salt which destabilizes proteins in solution and promotes protein solubility, the ability to form a strong network was interesting. Although SCN^- is a destabilizing anion, the T_d value at a concentration of 0.3 M was still high (93.6 ºC). A similar result has been reported for the 11S soy globulin (Damodaran, 1988). In a study of chaotropic anions on the release of insoluble membrane proteins, Hincha (1998) observed that the presence of chaotropic salts reduced the energy barrier for the dissociation of proteins from their binding sites on the membrane. In the present study, this reduction in the energy at the relatively low NaSCN concentration led to enhanced gel stiffness. Higher NaSCN concentrations were examined to further investigate the impact of this chaotropic salt. With 1 M NaSCN, the gel stiffness (G') was reduced significantly, although the relative elasticity (tan δ) was not affected, but with 3 M NaSCN, gel formation was inhibited (Fig. 6.1). At the higher concentrations, the binding of SCN^- resulted in a change in overall protein charges such that the structure was destabilized (von Hippel & Schleich, 1969; Damodaran & Kinsella, 1981) and gel formation was inhibited.

6.4.2. Effects of various reagents on non-covalent bonds

Various molecular forces, including hydrogen bond, hydrophobic and electrostatic interactions, tend to be disrupted by most chaotropic agents such as detergents, urea, or GuHCl (Sood & Slattery, 2003). To investigate these non-covalent bonds that contribute to the gel formation, pea protein isolate was dispersed into various reagents (GuHCl, PG, and Urea) solution containing 0.3M NaCl prior to heat treatment.

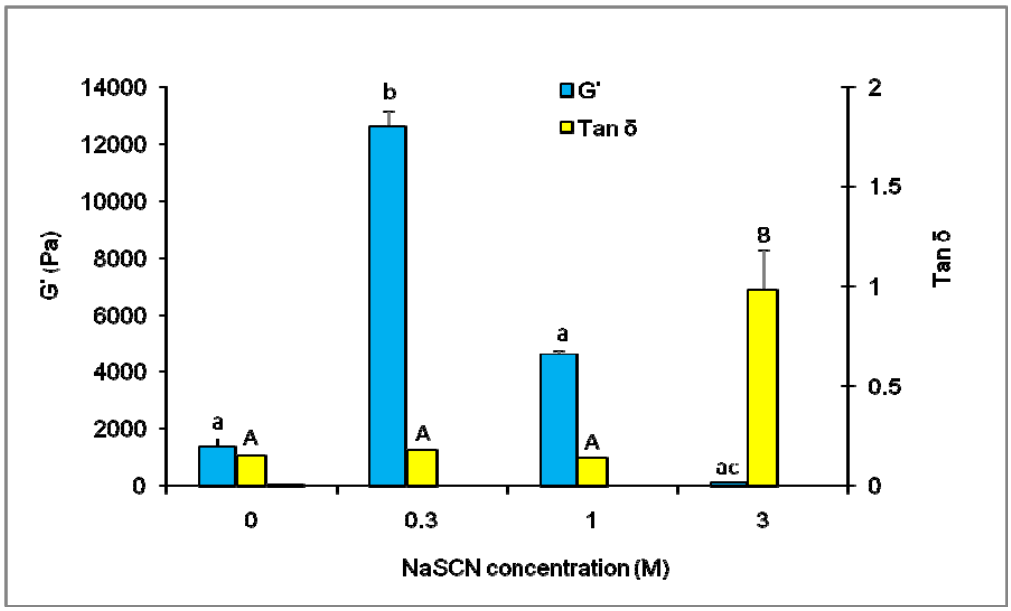

Fig. 6.1 Effect of different concentration NaSCN on gelation properties of 14.5% (w/v) pea protein isolate dispersion contains 0.3M NaCl, at pH 5.65.

6.4.2.1. Effect of Guanidine hydrochloride (GuHCl)

GuHCl is a strong ionic denaturing agent (Tanford, 1968), which weakens hydrophobic interactions and inhibits hydrogen and ionic bonds (Table 6.1). Tanford (1968) concluded that GuHCl gave the most extensively unfolded state, in which the protein molecules are devoid of their native conformation and behave as random coils. GuHCl is a more effective denaturant than urea, unfolding proteins at two to three times lower concentrations than urea (Greene & Pace, 1974), and GuHC1 is chemically stable, while urea slowly decomposes to form cyanate and ammonia.

As expected, the addition of GuHCl to the pea protein isolate used in this study resulted in protein denaturation, as evidenced by gradual decrease in ΔH up to 1.0 M GuHCl and no measureable structure change with 3 M GuHCl (Table 6.3). The higher T_d

values with 0.3 and 1.0 M GuHCl are an indication that the more stable structural components were retained, despite the lower enthalpy values. While the T_d values at 0.3 and 1.0 M GuHCl were low enough for the protein to unfold during gel preparation, there was a gradual decrease in the G' values with higher levels of GuHCl. At a concentration of 3.0 M GuHCl, gel formation was inhibited. This evidence supports the need for hydrogen and ionic bonds in gel formation.

Table 6.3 Effect of guanidine hydrochloride (GuHCl) concentration on the rheological properties of pea protein gels at 1 Hz and thermal denaturation of pea protein isolate. The concentration of pea protein isolate dispersion was 14.5% at natural pH 5.65 (all samples contained 0.3M NaCl).

GuHCl Concentration (M)	G'* (Pa)	Tan δ*	T_d* (°C)	ΔH* (J/g protein)
0	4516±188 [a]	0.1678±0.00 [a]	86.2±0.1[a]	15.81±0.0 [a]
0.3	619.5±113.8 [b]	0.108±0.00 [a]	93.04±0.09 [b]	10.92±0.26 [b]
1.0	203±51.6 [c]	0.249±0.02 [a]	93.24±0.23 [b]	9.08±0.13 [c]
3.0	0.08±0.04 [c]	15.09±5.86 [b]	–**	–**

* Mean±SD of duplicate.

* Column values followed by the same superscript letter are not significantly different ($p<0.05$).

** No denaturation was observed at this concentration.

6.4.2.2. Effect of Propylene glycol (PG)

PG may disrupt hydrophobic forces and enhance hydrogen bonds and electrostatic interactions by lowering the dielectric constant of solvent, and reducing the energy barrier to protein-protein interaction enough to enable structure formation (Utsumi & Kinsella, 1985a). Our results showed that the gel stiffness of pea protein gradually

increased with the increasing amount of added PG but the relative elasticity (tan δ) did not change (Fig. 6.2). This would suggest that hydrogen bonds and electrostatic interaction play a prominent role in determining the stiffness of pea protein gel. These results are consistent with the suggestion that electrostatic interactions are important in the formation of elastic gels, and suggest that hydrogen bonding complements electrostatic interactions in pea protein gels.

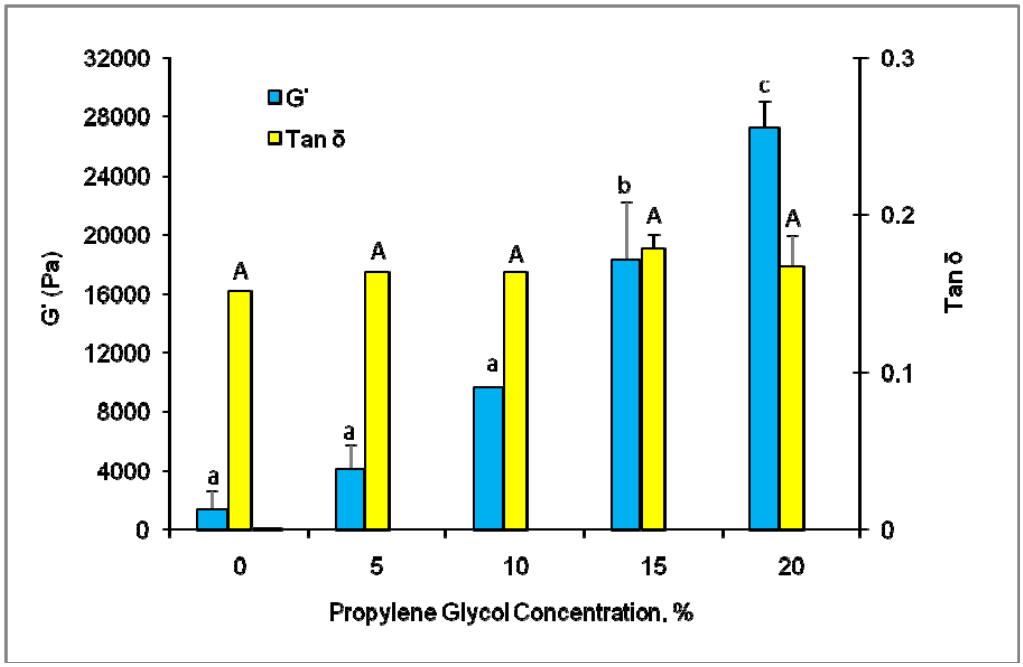

Fig. 6.2 Effect of different concentration PG on gelation properties of 14.5% (w/v) pea protein isolate dispersion contain 0.3M NaCl, at pH 5.65.

6.4.2.3. Effect of Urea

Zou et al. (1998) indicated that urea binds to amide groups through hydrogen bonds, decreasing the hydrophobic effect through dehydration of the protein molecule, and pointing out that hydrophobic groups and hydrophilic groups are involved in the

denaturation caused by urea. Walstra (2003) also reported that the denaturing effect of urea is caused by a dehydration of peptide bonds which were bound by urea also weakens hydrophobic interactions. In Fig. 6.3, G' was unaffected when the urea concentration was raised to 2 M, but decreased dramatically at 5 M and no gel was formed at 8 M. Urea denatured pea protein severely by breaking down hydrogen bonds and hydrophobic interactions, preventing network formation. This reduction in G' of pea protein gel confirms the involvement of hydrogen bonds and/or hydrophobic interactions in gel networks. Tanford (1968) indicated that urea is a strong denaturing agent which can induce extensively unfolded state, in which the protein molecule behaves like a random coil. The denatured states obtained by other denaturants are "intermediate" states between native and urea denatured states (Tanford, 1968).

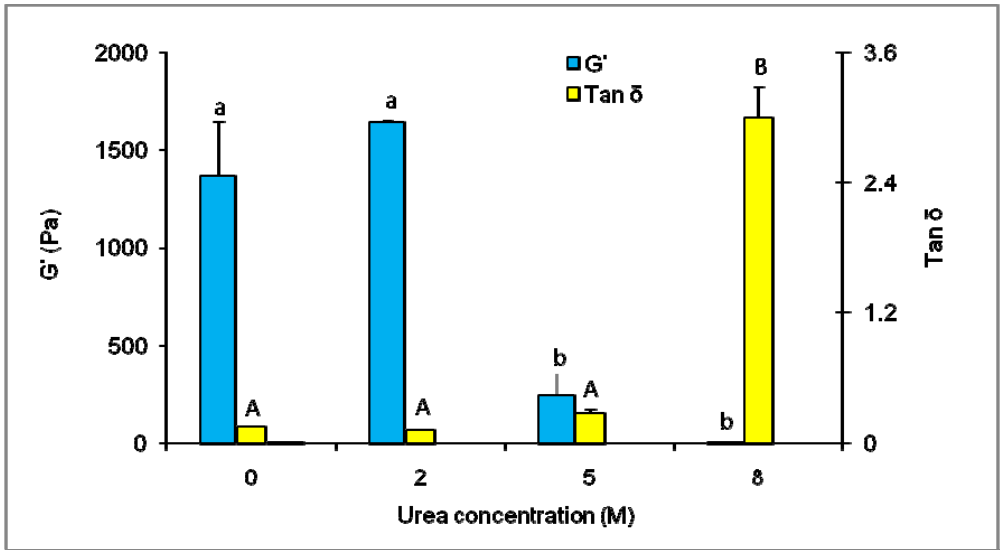

Fig. 6.3 Effect of different concentration urea on gelation properties of 14.5% (w/v) pea protein isolate dispersion contain 0.3M NaCl, at pH 5.65.

Hydrogen bonds, hydrophobic interactions, and electrostatic interactions are all believed to be important in the overall balance of attractive and repulsive forces contributing to network formation.

6.4.3. Effect on covalent bond

6.4.3.1. Effect of DL-Dithiothreitol (DTT)

DTT is often used to reduce the disulfide bonds of proteins and to prevent intra- and intermolecular disulfide bonds from forming between cysteine residues (Rüegg & Rudinger, 1977). Although DTT is a disulfide reducing agent, our results showed that its addition to pea protein dispersions at low concentrations (0.05 ~ 0.15 M) did not disrupt protein gel stiffness, as there was no significant change in G' values with increasing levels of DTT (Fig. 6.4). However, at DTT concentrations of 0.05 and 0.1 M, the tan δ values were significantly higher than the control, suggesting a decrease in the relative elasticity of the networks. Thus disulfide bonds do not appear to play a key role in the pea protein gel formation, but may affect gel characteristics.

6.4.3.2. Effect of 2-mercaptoethanol (ß-mercaptoethanol, 2-ME)

By competing for sulfhydryl group, mercaptoethanol can reduce disulfide bonds of protein (Wang & Damodaran, 1990). As a result, the effect of adding 2-ME reflects the contribution of disulfide bonds to pea protein gel network formation and maintenance. Results were similar to those with DTT in that 2-ME had no effect on the G' value for the pea protein gels and tan δ values were higher in the presence of 2-ME (Fig. 6.5) further

supporting the observation that disulfide bonds do not play a major role in pea protein gel formation.

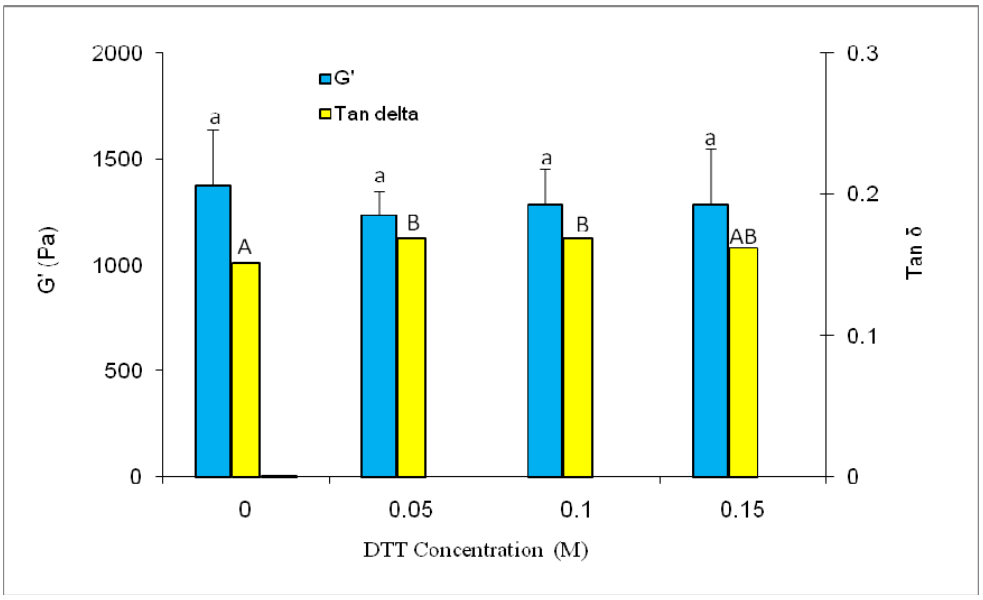

Fig. 6.4. Effect of different concentration DTT on gelation properties of 14.5% (w/v) pea protein isolate dispersion contain 0.3M NaCl, at pH 5.65.

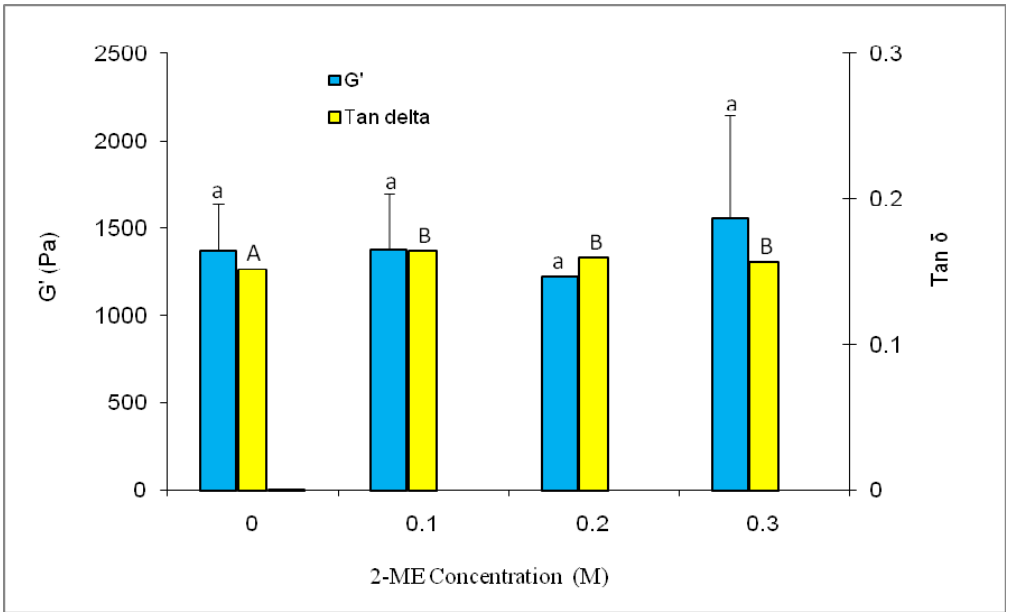

Fig. 6.5. Effect of different concentration 2-ME on gelation properties of 14.5% (w/v) pea protein isolate dispersion contain 0.3M NaCl, at pH 5.65.

6.4.3.3. Effect of N-ethylmaleimide (NEM)

NEM is a reagent which can react with sulfhydryl groups to form a stable alkyl derivative; preventing the formation of disulfide bonds between protein molecules (Creighton, 1993). Our results showed that NEM concentration of 5 mM and less had no impact on gel characteristics. With 100 mM NEM, the G' values were greater than without NEM or at lower NEM concentrations and tan δ values were also higher than the control (Fig. 6.6). Hua et al. (2005) investigated the effect of NEM on the gel forming ability of different varieties of soybean and obtained similar results. A similar phenomenon involving myofibrillar protein was attributed to cleaving of inter- and intra-molecular disulfide bonds thus facilitating protein unfolding and increasing the exposure of reactive groups involved in hydrogen bonding and ionic and hydrophobic interactions (Ustunol et al., 1992). This may also be the case for pea proteins at an NEM concentration of 100 mM, where a stronger less elastic gel is formed.

It was noted that when the sample was cooled slowly (0.5 °C/min), the effect of adding NEM was more pronounced. G' values for a sample with 20mM NEM were greater than those without NEM, whereas no difference could be seen when cooling at 2 ºC/min (Fig. 6.7). O'Kane et al. (2004b) indicated that the main forces involved in the formation of gel structure of pea legumin protein are hydrogen bonds, hydrophobic interaction, and disulfide bonds at slower cooling rates (1 °C/min heating and 0.2 °C/min cooling). Our result was consistent with their observation. They explained that disulfide bonds became involved in the gel network since the slower cooling rates provide time to react and contribute to gel stiffness.

Probably due to lower concentrations of DTT and 2-ME used in pea protein gelation, no change in G' value was observed for both of them in the whole concentration ranges. However, pea protein gelation with inclusion of NEM was different, when NEM concentrations were lower than 50 mM, there was no difference in G' values; when NEM concentration was 100 mM, significant difference was observed between 50 and 100 mM, indicative of cleaving inter- and intra-molecular disulfide bonds therefore enhancing protein unfolding and increasing the exposure of reactive groups involved in hydrophobic interactions and hydrogen bonding.

Overall, under normal heating and cooling (1 ºC or higher), disulfide bonds do not contribute to gel formation and have only a minor impact on gel characteristics. This is consistent with the results of O'Kane et al. (2004b), who indicated that disulfide bonds had minimum involvement in the network formation of isolated pea legumin proteins.

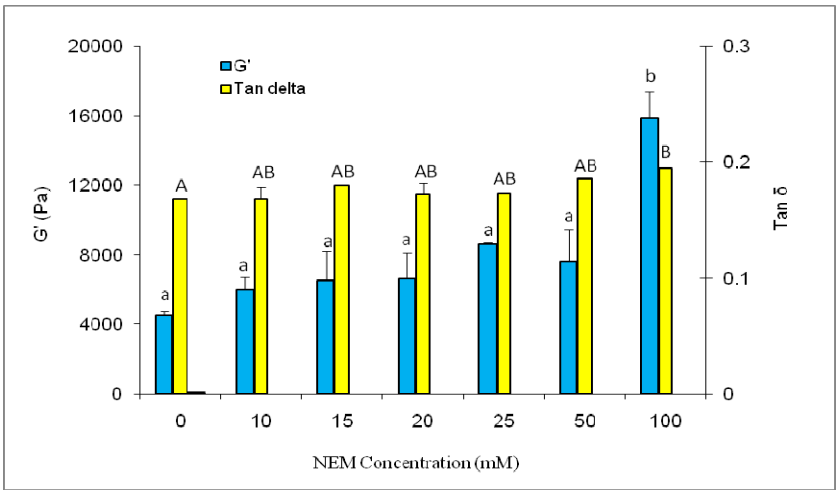

Fig. 6.6. Effect of different concentration NEM on gelation properties of 14.5% (w/v) pea protein isolate dispersion contain 0.3M NaCl, at pH 5.65.

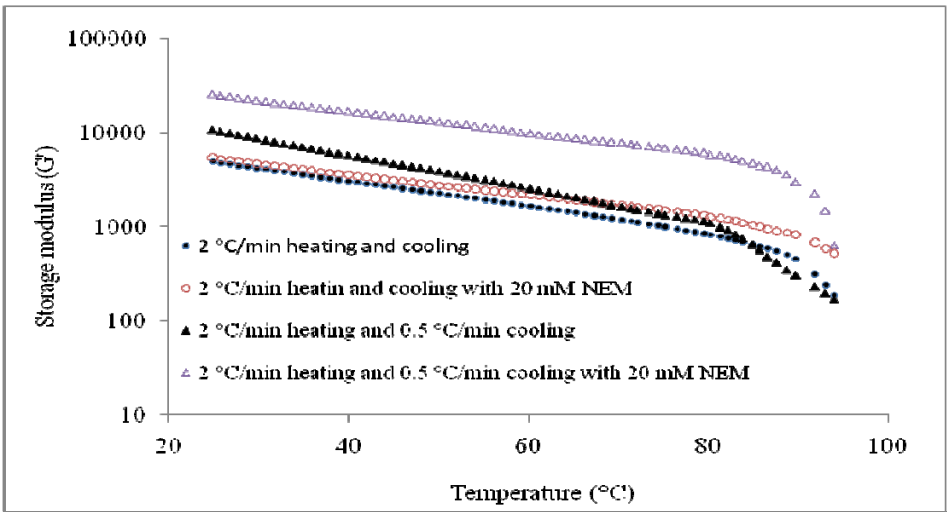

Fig. 6.7. Effect of the addition of 20 mM NEM on the development of storage modulus (G') during heating and cooling phase of 14.5% (w/v), 0.3 M NaCl, pea protein dispersion. Heating phase of different treatments is essentially identical with each other thus not shown.

6.4.4. Effect of reheating and recooling

Heating and cooling curves monitored structure development of pea protein isolate during processing. Rheological measurement indicated that structure development begins at temperatures of about 85~87 ºC during the heating phase (Fig. 6.8). Hydrophobic interactions are endothermic and as a result, are stronger at high temperatures and weaker at low temperature (opposite to that for hydrogen bonds) (Damodaran, 1996). Therefore, structure formation at these high temperatures probably involves hydrophobic interactions.

A reheating and recooling process was used to investigate the contribution of hydrogen bonds to the formation of pea protein gel. Upon reheating, the G' values steadily declined as the temperature rose to 95 °C, with a rate of decline almost the same as the rate of increase in G' during cooling and reached the same level as G' of pea

protein dispersion which was heated to 95 °C (Fig. 6.8). This indicated that development of gel rigidity during the cooling phase was thermally reversible. Since hydrogen bonds are weakened with increasing temperature, the thermal reversibility provided further evidence for the contribution of hydrogen bonds to gel rigidity (Fig. 6.8).

During the final recooling phase, hydrogen bonds are again believed to be important in gel stiffening. Since hydrogen bonding is favored at low temperatures, the gradual increase in G' values during the cooling phase is again attributed to this molecular force. However, G' values could not reach the same level as obtained during the first cooling stage, probably because during recooling, not all the hydrogen bonds disrupted in the reheating stage could recover.

In light of the fact that hydrophobic interactions are weakened and hydrogen bonds strengthened at lower temperatures, the gradual increase in G' values during cooling and recooling indicates hydrogen bonds played a more important role than hydrophobic interactions on rigidity of gel network.

Heating pea protein pretreated with DTT and 2-ME indicated that disulfide bonds were not involved in the gelation process, however inclusion of NEM at higher level, plus slower cooling rate (0.5 ºC/min with the addition of NEM; Fig. 6.7) provided evidence that disulfide bonds were involved. Léger & Arntfield (1993) indicated that if a gel fails to melt upon heating this is usually indicative of the presence of covalent bonds. This situation was seen for pea protein gel reheated to 95 ºC (Fig. 6.8) as a weak gel structure was retained. In this case, disulfide bonds were probably involved in the stabilization of the gel structure.

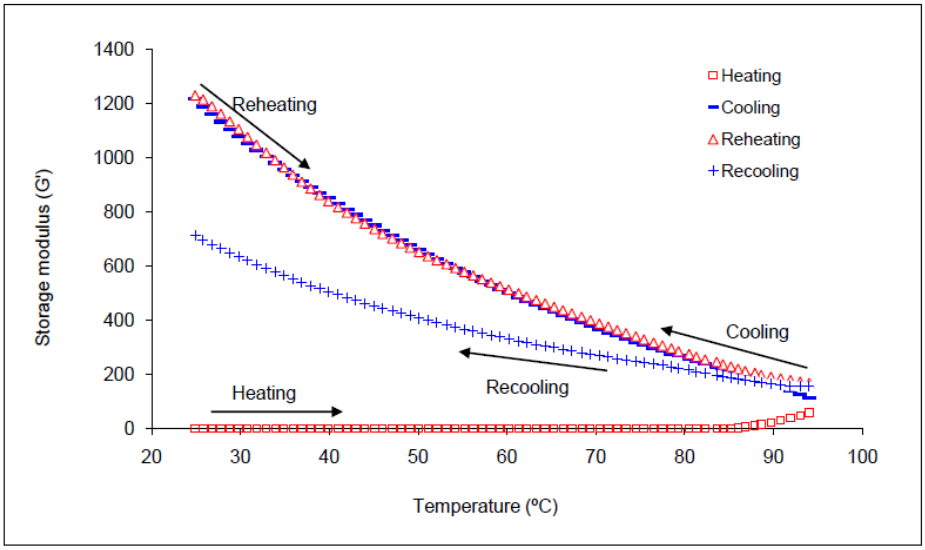

Fig. 6.8 Effect of reheating and recooling on gelation properties of G' of 14.5% (w/v) pea protein isolate dispersed in 0.3M NaCl at natural pH (5.65).

6.5. Conclusion

Our results indicated that for pea protein isolated by salt extraction and precipitation by dilution, non-covalent bonds played a key role in pea protein gel formation, while disulfide bond prevention or reduction did not influence gel stiffness. The presence of disulfide bonds was supported by reduced gel stiffness when using slow heating and cooling rates in the presence of NEM and incomplete melting upon reheating the gel. This role was minor and under normal heating conditions was shown to affect gel elasticity but not gel stiffness. While electrostatic and hydrophobic interactions contributed to the initial structure development during gel formation. Hydrogen bonds were responsible for strengthening the gel during cooling.

Chapter 7: Gelation properties of salt extracted pea protein isolate catalyzed by microbial transglutaminase cross-linking (Sun, X. D. and Arntfield, S. D. 2011. *Food Hydrocolloids*, 25, 25-31)

7.1. Abstract

Gelation is a fundamental functional characteristic of plant proteins. In this paper, a salt-extracted pea protein isolate (PPI) was mixed with microbial transglutaminase (MTG) to produce gels and the gelation properties were studied. When the MTG level increased, the magnitude of both the G' and G" moduli also increased, which means the gel stiffness increased. A second order polynomial equation was used to describe the relationships between the G', G" modulus and MTG level. It was found that with increased heating and cooling rate at the same MTG level, G' and G" tended to decrease, resulting in a weaker gel. This was attributed to the rearrangement time of pea protein molecules; slower heating and cooling rates enabled protein molecules to have more time to rearrange and therefore form a stronger gel. At the same MTG level, higher pea protein concentration resulted in higher G' and G" values and a power law relationship was found between G' and pea protein concentration or G" and pea protein concentration. Frequency sweep data of PPI show that the MTG treatment resulted in higher G' values and lower tan delta values, indicative of a stiffer, more elastic gel. The minimum gelation concentration was found to be 3% (w/v) with 10U MTG treatment, lower than 5.5% required when no MTG was present. When compared to PPI and soy protein isolate (SPI) with and without 10U MTG treatment, the gel stiffness of PPI with MTG was more than that of SPI with MTG treatment, whereas the opposite was true without the MTG

treatment. SDS-PAGE showed that at the same pea protein concentration, higher MTG level induced more cross-linking as fainter bands were seen on the gel and there was a shift in the relative intensities of the bands in the molecular weight range of 35~100kDa.

7.2. Introduction

Pea is an important cash plant in Canada. In recent years, pea has been processed into pea flour, pea protein isolate (PPI), pea starch, and pea fiber, etc. The application of pea as a protein source is still limited because of its relatively weak functionality as a food ingredient.

Soy proteins are used in foods as functional and nutritional ingredients or as a substitute for animal-derived proteins from milk, meat and eggs (Qi et al, 1997). Protein isolates from soybean have dominated the market for many years; however, recently a trend for using alternative protein isolates with similar functional and nutritional properties has emerged (Marcone et al., 1998). A potential alternative plant protein that could be used is pea (*Pisum sativum* L.). Soy protein contains two major globulin proteins: legumin and vicilin. Pea protein also contains these two major globulin proteins; therefore, it may be expected that they would have similar functional properties.

Globular proteins from various sources (in the form of isolates) play important roles in many foodstuffs, both because of their nutritional value and of their contribution to food texture (van Kleef, 1986). These textural contributions come from the network structures created by the proteins. Since gelation is one of the most important functional properties of the globular proteins used to modify food texture (Ikeda & Nishinari, 2001), it is essential to understand which factors determine the gel network and how they are

affected by processing parameters. Such an understanding would enable better control of food texture.

Cross-linking is believed to be an important way to improve gel formation. Both chemical and enzymatic treatments can influence gel formation in proteins. Compared to the chemical treatment, enzymatic cross-linking of food proteins is considered to be more acceptable to consumers. Transglutaminase (TG) is an enzyme often used for the cross-linking of food proteins (Aguilera & Rademacher, 2004; Haard, 2001).

Transglutaminase (glutaminyl-peptide:amine λ-glutamyltransferase, E.C. 2.3.2.13) is widely distributed in nature. TG can modify proteins by catalyzing the acyl transfer between a λ-carboxyamide of a peptide/protein bound glutamine and lysine forming an ε-(λ-glutamyl) lysine [ε-(λ-Glu) Lys] cross-link (Kuraishi et al., 2001). TG catalyzes conversion of soluble proteins into insoluble high molecular weight polymers through formation of covalent crosslinks (Motoki et al, 1987; Nino et al, 1985).

A prerequisite for cross-linking with TG is the availability of lysine and glutamine residues, which through the action of TG become covalently bound forming either inter- or intra molecular ε-(γ-Glu)-Lys cross-links (De Jong & Koppelman, 2002; Nielsen, 1995). Kang et al. (1994) showed that the amount of surface lysine and glutamine residues were correlated with the amount of cross-links formed.

TG can be extracted from both animal and microbial sources. However, the application of mammalian TG in food processing on an industrial scale is restricted due to its poor availability, complicated separation and purification procedures as well as the

requirement for calcium. Compared to animal source TG, microbial transglutaminase (MTG) is easily obtained by microbial fermentation and it can be produced in large enough scale for commercial use (De Jong & Koppelman, 2002).

Many plant proteins are globular (Nielsen, 1995). It is therefore expected, that any treatment which opens the protein structure and makes the buried reactive groups more available, would improve the TG cross-linking (Nielsen, 1995). Thus partial denaturation of protein, as would result from a heat treatment, could increase the level of TG cross-linking.

Controlled denaturation could open globular protein structure, exposing more functional residues for crosslinking. This has the potential to form a relatively stronger gel compared to a commercial pea protein isolate, where the alkaline extraction, isoelectric precipitation and spray drying have been shown to cause extensive denaturation and reduced gelation ability. As reported previously, a low denaturation salt-extraction method was developed to extract pea protein isolate from pea flour and the gelation properties of this isolate were also studied. We found that gelation properties of salt-extracted pea protein were improved compared to commercial pea protein isolate. Studies on gelation of pea legumin and vicilin have been reported by Owusu-Ansah & McCurdy (1991); Bacon et al., (1990); Bora et al., (1994); O'Kane et al. (2004a, 2004b, 2004c); but none of these studies worked on the gelation properties of salt extracted pea protein isolate (PPIs) and none looked at transglutaminase cross-linking. The objectives of this research are to study the gelation characteristics of various PPIs and commercial soy protein isolate (SPIc) catalyzed by MTG cross-linking.

7.3. Materials and methods

7.3.1. Commercial pea flour, PPIs and SPIc

Commercial pea flour and pea protein isolate were donated by Nutri-Pea Ltd. (Portage la Prairie, MB, Canada). The flour is made from Canadian yellow pea using air classification. The commercial pea protein isolate (PPIc) prepared by acid extraction and isoelectric precipitation, contained 82% of protein. Salt-extracted pea protein isolate, extracted from commercial pea flour (protein content 19.93 ± 0.16%), contained 81.91 ± 0.37% protein as determined by Kjeldahl (AACC, 1982) using a N to protein conversion factor of 5.7. Commercial soy protein isolate (SPIc) (PRO-FAM 974) was obtained from Archer Daniels Midland Company (ADM) (Decatur, IL, U.S.A.) and protein content was 90%.

7.3.2. Extraction of PPIs

Salt-extraction method of pea protein isolate is described in a previous paper (Sun & Arntfield, 2010). Sodium chloride (0.3 M) was employed to extract pea protein from pea flour (pea flour: sodium chloride solution = 3:10, w/v), the soluble pea proteins were then separated by centrifugation (5000 rpm, 20 min) and precipitated by dilution in cold water (supernatant:water = 1:2, v/v). The protein was resuspended in water and unwanted salt was removed from pea protein concentrate by dialysis. The desalted protein isolate was then freeze dried.

7.3.3. MTG treatment

The pea protein isolate was mixed with 0.3M NaCl (Fisher Scientific, Ottawa, Canada) to obtain dispersions of a series desired concentration. The pea protein was

dispersed in 0.3 M NaCl as a good gel formation has been reported at this salt concentration compared to other salt concentrations. The samples were mixed using a Vortex-Genie Mixer (Scientific Industries Inc., Bohemia, N.Y., USA) for 1 min to ensure thoroughly distribution of pea protein dispersion. The pH of dispersion was in the range of 5.65-5.66.

A microbial Ca^{2+} independent MTG (Activa TI, Ajinomoto, Paramus, USA) were used to crosslink pea protein. The *Activia TI* MTG enzyme contains 100 U of activity per 1 *g* of powdered product. MTG activity of 10U/mL solution was prepared by dissolving 1 g of MTG in 10mL distilled water. According to experimental design, an appropriate aliquot of the enzyme solution was then added to the pea protein isolate dispersion. After mixing using a Vortex for 10 sec, it was loaded to the rheometer.

7.3.4. Rheology

An AR2000 rheometer (TA Instruments, Newcastle, Del. U.S.A.) was used to test rheological properties of pea proteins. One ml of pea protein isolates dispersion with or without MTG was transferred to the lower plate of the parallel plate geometry. The upper plate was lowered to a gap width of 1.00 mm. To avoid water losses during measurement, a solvent trap cover was used to prevent sample drying during heating. In this way, a water-saturated atmosphere was maintained at the surface of the sample.

Samples were incubated at 40°C for 30 min after loading. Samples were then heated and cooled over a temperature range of 40-95-25°C at a rate of 2°C /min, followed by a frequency sweep (0.01-10Hz) at 25°C. Rheological data (storage modulus (G') and loss modulus (G")) were collected during the heating and cooling steps as well as during

frequency sweep with a thermal equilibrium time of 10s. Samples were run at least in duplicate, and a representative sample has been presented.

7.3.5. Electrophoresis

Following rheological analysis, gels were removed from the rheometer and then freeze dried (Genesis SQ Freeze Dryer, Gardiner, NY, U.S.A.). To evaluate subunits in the protein gels, the method of Aluko & McIntoch (2001) was followed with minor modifications. Samples of 5 mg were weighed into a microcentrifuge and dissolved in 1 M Tris-HCl (T-1503, Sigma, St.Louis,USA) sample buffer (5% w/v) at pH 8 containing 10% SDS (L-3771, Sigma, St.Louis, USA), 5% 2-mercaptoethanol (M-7154, Sigma, St. Louis, USA) and 0.01% Pyronin Y (P-6653, Sigma, St. Louis, USA). Samples were boiled for 10 min and then vortexed (Vortx Genie 2, Scientific Industries Inc., Bohemia, USA) to promote dissolution. The sample was then centrifuged at 14,000 × g (Biofuge A, Canlab, West Germany) to settle out any remaining particulate matter and 7µL of the supernatant were applied to each well of a gel containing 4% acrylamide stacking gel and 12% acrylamide separating gel. The standard (SDS-PAGE Molecular Weight Standards, Broad Range, Bio-Rad, Hercules, USA) was prepared as per manufacturers' instructions and 5 µL were loaded into one well. Gels were run for 2h and 20 min at 10 amp per gel (Mini-ProteanR 3 Cell, Boi-Rad, Hercules, USA). A staining solution consisting of 0.08% (w/v) Commassie brilliant blue G-250 (B-1131, Sigma, St. Louis, USA), 10% (w/v) ammonium sulfate (ACS 093, BDH Inc., Toronto, Canada) and 2.5% (w/v) phosphoric acid (Fisher Scientific, Ottawa, Canada) was used to visualize the protein bands. A 20% ammonium sulfate (ACS 093, BDH Inc., Toronto, Canada) solution was

used to destain the gels. The gel was scanned (GE Image Scanner, UTA-1100, Taiwan) and Image Quant^{TM} TL software was used to analyze band density and molecular weight.

7.3.6. Minimum gelling concentration

Minimum gelling concentration was determined by a procedure adapted from the method of O'Kane et al. (2005) with a slight modification. Five mL protein dispersions were prepared using 2-5% (w/v) of PPIs, in 0.3 M NaCl buffer. Samples were mixed with 10U MTG and heated to 40°C in sealed test tubes (10 mm diameter × 75 mm in length) to avoid evaporation in a water bath for 30 min to allow MTG to react. Samples were then heated to 95°C in a water bath and kept for 10 min at 95°C, prior to cooling to room temperature for 1 h, and storing at 4°C overnight. The next day the tubes were inverted and the samples that did not flow were considered to have gelled, and the lowest concentration where this occurred was the minimum gelling concentration.

7.3.7. Statistical analysis

All data were analyzed for significant differences using one way ANOVA, and minimum significance was set at the 5% level (P<0.05) using Tukey's test by GraphPad InStat software version 3.06 (GraphPad Software Inc. La Jolla, CA, USA).

7.4. Results and discussion

As rheograms obtained from different measurements (replicates) were the same, for ease of presentation, only one set of the data is included.

7.4.1. Gel formation with and without MTG

Gelation of pea protein results from the transformation of a viscous polymer solution to a 3-dimensional elastic network. Gel formation can be monitored by dynamic rheological parameters. Although this test has not always been shown to correlate with sensory texture, the method is suitable for measurement of subtle changes associated with the gel forming phenomenon (Hamann, 1987). Changes in the storage modulus (G') can be used to follow gelation of pea proteins. Rheology for gelation of proteins and other systems has been used for decades as indicated by the dates of the references given here. The temperature at which G' becomes greater than the loss modulus G" is identified as the initiation of gelation, the gel point and is commonly referred to as the G'-G" crossover (Ikeda et al., 2001; Stading & Hermansson, 1990; Ross-Murphy, 1995; Yoon et al., 1999).

The initial evaluation of G' for the MTG treated sample was made immediately after incubating the samples for 30 min at 40 ºC (Fig. 7.1). At this point, G' for the sample containing MTG was already greater than that without the MTG treatment which had been heating from 25 ºC to 40 ºC at 2 ºC/min. This is most likely due to the formation of larger aggregates and also probably indicates the three-dimensional network began to form during the MTG treatment.

O'Kane et al. (2004c) indicated that with cooling networks develop further and are strengthened by the formation of many short-range interactions such as hydrogen bonds. It was the same situation with or without the addition of MTG, since G' and G" continued to increase steadily during the cooling phase (Fig. 7.1). MTG induced cross-

linking only happened at low temperature in the heating phase before the enzyme was inactivated.

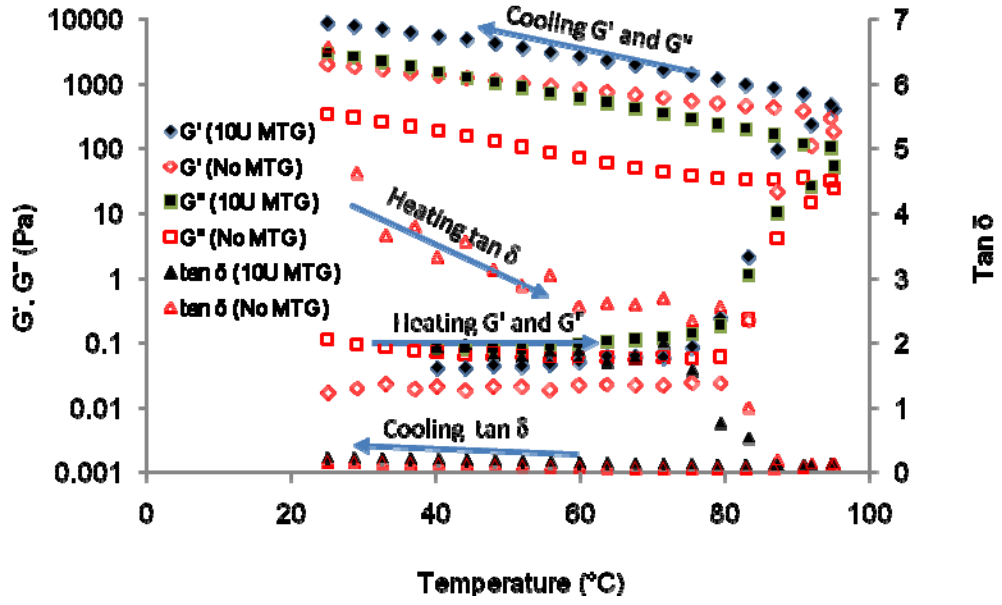

Fig. 7.1 Gel formation pattern of PPIs with MTG (10U/g protein) and without MTG at PPIs concentration 14.5% (w/v), 0.3M NaCl, pH 5.65.

In the present experiments, all the pea protein samples mixed with MTG had significantly higher G' than G" values when compared with the samples without MTG addition (Fig. 7.1). G' values increased continuously throughout the heating and cooling process. The increase in storage and loss modulus of gels during heating has been ascribed to the presence of hydrophobic forces contributing to gelation (Mleko & Foegeding, 2000).

7.4.2. Effect of heating and cooling rates on gels formation with MTG

With increasing heating and cooling rates, G' values decreased, G" values also decrease (data not shown), and tan δ values increased (Fig. 7.2), indicating lower heating and cooling rates induced stronger gel networks. It was concluded by Arntfield & Murray (1992) that the slower the rate of aggregation relative to denaturation, the more fine-stranded and ordered is the resultant gel network. O'Kane et al. (2004c) indicated that slow cooling could maintain the protein in its unfolded state for a longer time, slowing down the reactivity of the exposed residues, and enabling more optimal interactions to happen. In addition, slow cooling gave the opportunity for disulfide bonds to become involved in the gel network, thus resulting in additional strength (O'Kane et al., 2004c).

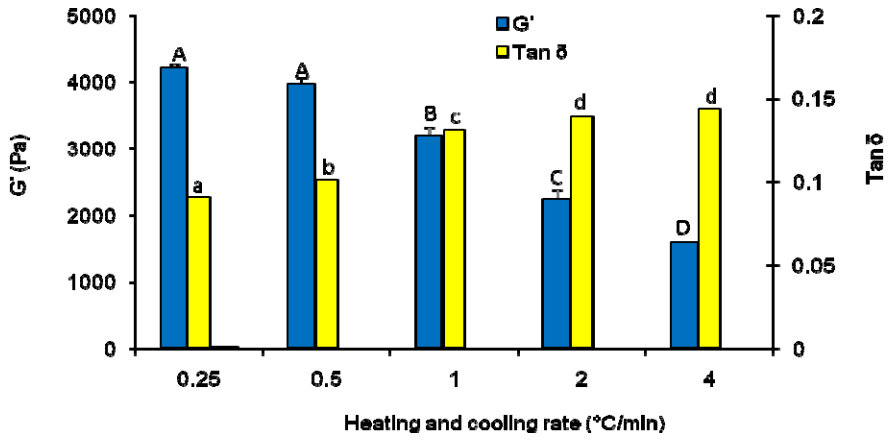

Fig. 7.2 Changes in the rheological parameters of PPIs dispersions as function of heating and cooling rate at 1 Hz sweep frequency. PPIs concentration 10.5% (w/v), 0.3M NaCl, 10U MTG.

Linear relationships were used to describe the impact of heating and cooling on G', G" and tan δ; the following equations were obtained:

$G' = -695V + 5122$, $R^2 = 0.97$

$G'' = -28.7V + 444$, $R^2 = 0.58$

$\tan \delta = 0.01V + 0.08$, $R^2 = 0.92$

Where V represents heating and cooling rate.

In comparison, no linear relationships were obtained between G', G" and heating and cooling rates when no MTG was added. Lack of linear relationships between G', G" and heating and cooling rates on fababean vicilin and pea protein was also reported by other workers (Arntfield & Murray, 1992; O'Kane et al., 2005).

7.4.3. Characteristics of gels produced with and without MTG

7.4.3.1. Variation in rheological parameters due to oscillatory frequency

There was a slight increase in G' with an increase in frequency (Fig. 7.3). For a totally elastic system, the G' values should be independent of frequency (Arntfield et al., 1989). Therefore, the slight dependence on frequency reflected the viscoelastic nature of the network. In a strong gel, the molecular rearrangements within the network are much reduced over the time scales analyzed, G' is higher than G" throughout the frequency range, and G' is almost independent of frequency (ω), whereas in weak gels there is a higher dependence on frequency for the dynamic moduli (Lopes da Silva & Rao, 1999).

The combination of the low tan delta values and high G' values with frequency are indicative of a strong gel network. In the present study, the frequency sweep of PPIs

gel with MTG showed higher G' and lower tan delta values than those without MTG treatment (Fig. 7.3), and thus produced a relatively stiffer network than the gel lacking MTG.

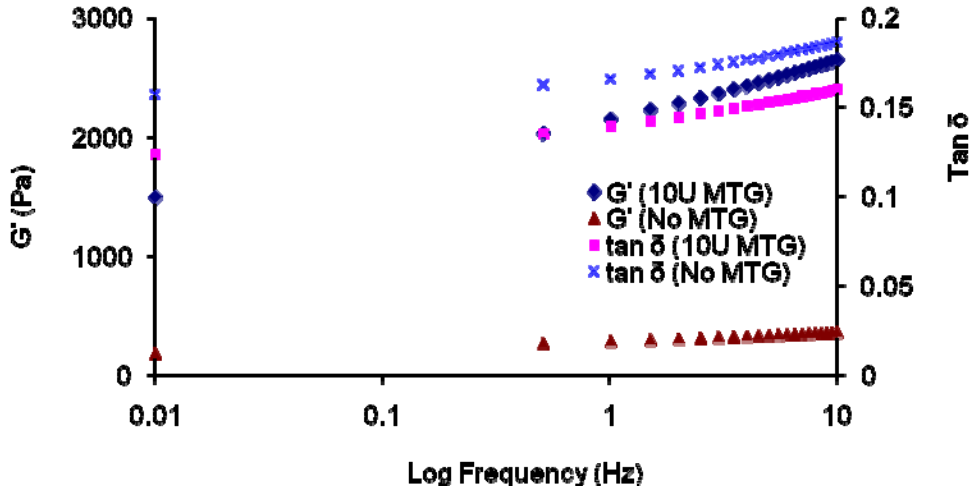

Fig. 7.3 Comparison of frequency sweep of gelation properties of PPIs with (10U) and without MTG at PPI concentration 10.5% (w/v) 0.3M NaCl, pH 5.65.

7.4.3.2. Minimum protein concentration for gelation

O'Kane et al. (2005) reported that the minimum concentration required for heat-induced gel formation at pH 7.1 is 16% (w/v) for pea protein isolates containing 20-28% legumin and 61-67% vicilins. In chapter 3, the minimum concentration to form heat induced PPIs gel was 5.5% (w/v). When MTG was included at 10U/g as in the current study, the minimum gelation concentration was 3% (w/v). The higher minimum concentration in the work O'Kane et al. (2005) was obtained with isolate prepared using an acid extraction; this would cause serious denaturation of the pea protein and decreased the gelation ability. The inclusion of MTG promoted additional cross-linking among

protein molecules and this increased the gelation ability of PPIs and as a result less protein is required to form a gel.

7.4.3.3. Effect of protein concentration

It is obvious that the storage modulus G' and loss modulus G" increase with increased PPI concentration at the same MTG level (Fig. 7.4). This is probably because when PPI concentration increases, the opportunities for cross-linking of PPI also increase. Therefore it can be concluded that higher PPI concentration induced formation of stronger gel.

As in previous studies (Arntfield, et al., 1990; van Kleef, 1986), increasing the protein concentration increased the magnitude of the G' and G" moduli. A power law relationship between G' and concentration or G" and concentration was obtained for pea protein when adding MTG (Fig. 7.4). In the study of Arntfield et al. (1990), a power law relationship was also found for G' and concentration or G" and concentration of vicilin. In the present study, the following equations were obtained:

$G' = 0.38C^{3.70}$, $R^2 = 0.98$

$G'' = 0.05C^{3.73}$, $R^2 = 0.97$

Where C represents pea protein concentration.

In the studies of van Kleef (1986) and Arntfield et al. (1990), the exponential factor of soy protein and vicilin for fababean for G' were 4.2 and 2.8 respectively, with the 3.7 value for pea protein in the present study falling between the two. The variations are probably due to different protein sources and treatment conditions. It was observed in the present study that there was a tendency for the PPIs to coagulate when adding MTG if

the protein concentration was over 14.5%. Hermansson (1979) indicated that if random aggregation and denaturation occur simultaneously or if random aggregation occurs before denaturation, the resulting network can be expected to show lower elasticity than if aggregation is suppressed prior to unfolding. Tombs (1974) also found that the higher the randomness of aggregation, the more likely that a coagulum is obtained instead of a gel. As a result the resulting network may contain a mixture of random aggregation and three dimentional network structures, with aggregation occurring upon the addition of MTG and network formation following a heat denaturation of the protein.

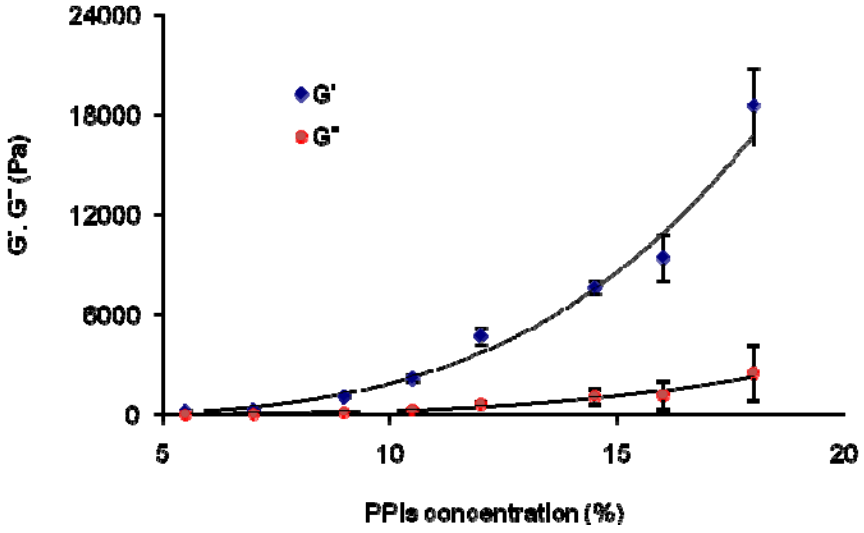

Fig. 7.4 Relationship between rheological parameters and PPIs concentration. 10U MTG, 0.3M NaCl, pH 5.65.

7.4.3.4. Effect of MTG concentration

Increasing the MTG level also increased the magnitude of complex modulus G^* ($G^* = (G'^2+G''^2)^{½}$) (Fig. 7.5). Second order polynomial equations between G^* and MTG level was obtained for pea protein, as shown in the following equations:

$G^* = 7.05U^2 + 52.9U + 489$, $R^2 = 0.9965$

Where U represents the MTG level.

The tan δ values decreased sharply between the MTG level of 1 to 5 U, but .remained relatively constant at MTG levels of 10-30 U. This indicated that a MTG level of at least 5 U was required to form a well crosslinked gel. The observed increase in G^* with increasing MTG levels shows that high levels of MTG produced stronger gels, though the relative elasticity did not change.

Changes in subunit composition as affected by MTG are shown in Fig. 7.6. A loss of high molecular weight polypeptides resulted from MTG cross-linking. Bands at 102, 74, 47, 41, 35 and 32 kDa grew fainter with the increasing amount of MTG, and almost completely disappeared when 30U MTG were included. The formation of high molecular materials is supported by an increase in the density at the point of application. This indicates that most of the PPI subunits cross-linked by MTG are in the molecular weight range of 35~74 kDa, which corresponds to pea vicilin and legumin acidic subunit (~41 kDa). Low molecular weight subunits (smaller than 25 kDa) were unaffected by MTG. Higher MTG levels produced more cross-linking. This clearly demonstrates that the increase in gel stiffness in the presence of MTG is due to the crosslinks formed between subunits.

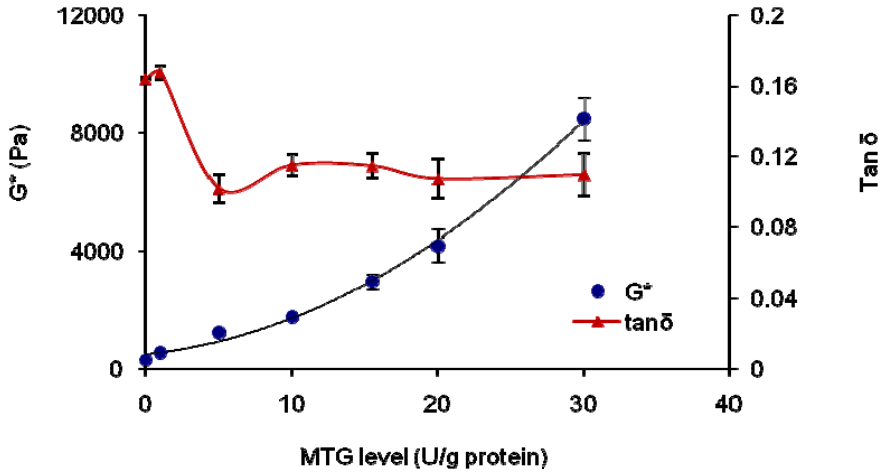

Fig. 7.5 Relationship of MTG level and G* and tan delta. PPIs concentration 10.5% (w/v).

As seen in Table 7.1, band relative intensity (% of total absorbance for lane) at molecular weights 41 and 35 kDa decreased with increased MTG level, indicating that these subunits preferentially crosslinked to form bigger molecules. For subunits at molecular weights 74 and 47 kDa, relative band intensities were higher with increasing MTG level. This occurred despite the observation that overall band intensities tended to decrease with increasing MTG level. This may be explained by the crosslinkings of relatively low molecular weight molecules such as at 35 and 41 kDa to form small amount of bigger molecules at 47 and 74 kDa. Alternately crosslinked subunits with molecular weight greater than 200 kDa formed by MTG catalyzed crosslinking could not enter stacking gel and thus accumulated on the top of the gel. This would decrease the total intensity, but because of the preferential loss of the 41+35 kDa subunits, the relative proportions of the 47+74 kDa increased.

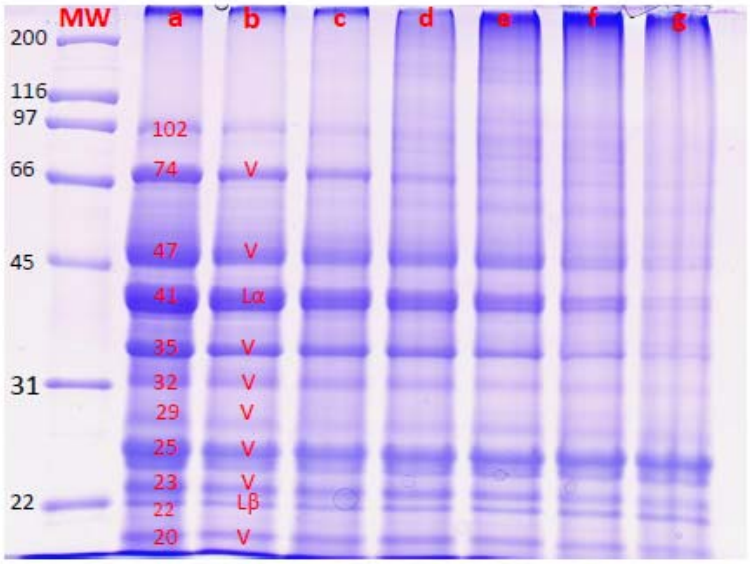

Fig. 7.6 SDS-PAGE composition of PPIs treated with varying levels of MTG. From left to right: (MW) molecular weight standard marker (kDa), (a) PPIs (powder, without heat induced gelation treatment), (b) 10.5% PPIs, No MTG (with heat induced gelation treatment (heated from 25 °C to 95 °C then cooled down to 25 °C at rates of 2 °C/min) (c) 10.5% PPIs, 1 U MTG (the following treatments were all used this procedure: first incubated at 40 °C for 30 min, then heated and cooled as described above), (d) 10.5% PPIs, 5 U MTG, (e) 10.5% PPIs, 10 U MTG, (f) 10.5% PPIs, 15.5 U MTG, (g) 10.5% PPIs, 30 U MTG. *V* represents pea vicilin proteins, L_α represents pea legumin acidic subunit, and L_β represents pea legumin basic subunit.

Table 7.1 Relative band intensity for SDS separation of pea protein gels with increasing MTG levels*

Band MW	a	b	c (1U)	d (5U)	e (10U)	f (15.5U)	g (30U)
102	5.2	3.2	3.8	5.1	6.5	6.6	5.8
74	2.5	3.1	5.8	8.4	6.6	9.5	9.3
47	11.9	9.2	11.1	12.5	12.6	13.2	13.7
41	17.3	15.6	15.2	14.8	12.7	12.0	11.6
35	11.6	10.4	9.9	9.8	9.6	9.1	8.3
32	2.0	1.7	1.6	1.5	2.6	2.7	2.6
29	6.9	5.3	4.8	4.6	5.8	4.9	5.1
25	15.6	15.0	12.0	10.5	11.1	11.7	15.1
23	13.9	24.9	23.9	21.9	21.2	20.4	19.2
22	13.1	11.5	12.1	11.1	9.6	9.8	9.2

* Refer to Fig. 7.6 to identify bands.

7.4.3.5. Comparison of PPI and SPI with and without MTG

When comparing gel stiffness for PPIs and SPIc without MTG, it can be seen that the G' value of PPIs (291.6 Pa) was smaller than that of SPIc (890.1 Pa), and tan delta value of PPIs (0.167) was greater than that of SPIc (0.124) (Table 7.2), which indicated that PPIs formed weaker gels than SPIc. The impact of MTG on PPIs, however, was greater than on SPIc, causing more crosslinking and a stronger gel than treated or untreated SPI. Schäfer et al (2007) also compared gelation and cross-link formation of a PPI and a SPI during MTG treatment. They found that the total ε-(λ-glutamyl) lysine content increased by ~200 μmol/100g for both PPI and SPI yet the gel PPI increased by 300% compared to 155% for SPI. A higher protein content for PPI and the positions of ε-

(λ-glutamyl) lysine cross-links generated in PPI were responsible for a greater impact on gelation in that study. In the current study, the PPI and SPI dispersions have the same protein concentration, so the greatly improved gelation properties for the pea protein was likely due to the position of the crosslinks.

Table 7.2 Gelation properties of PPIs and SPIc with and without MTG treatment. Protein concentrations of all samples were 10.5% (w/v), 0.3M NaCl. MTG level was 10U/g protein.

	PPIs with MTG	PPIs	SPIc with MTG	SPIc
G', Pa *	2221.5±95.4 [a]	291.6±4.2 [bc]	1581±532.4 [a]	890.1±41.4 [c]
G", Pa *	288.5±16.6 [a]	48.8±1.4 [b]	177.7±64.3 [ab]	111±9.1 [c]
Tan delta *	0.130±0.00 [a]	0.167±0.00 [b]	0.112±0.00 [a]	0.124±0.00 [a]

* Means±SD of duplicates.

[a~c] Row values followed by the same letter are not significantly different ($P<0.05$).

7.5. Conclusion

Pea protein alone can form a heat-induced gel. Through the use of MTG to form cross-links among pea protein polypeptide chains, both the strength and elasticity of the gel have been enhanced. With 10 U MTG, the gel stiffness (G') was 8 times higher than the untreated sample and higher MTG levels resulted in even stronger gels, as exponential relationships between G' or G" and MTG level were obtained. As expected pea protein concentration also affected gelation properties, and generally higher protein concentrations also produced stronger gels at a given MTG level. A power law relationship between G' or G" and concentration was obtained. At the same MTG level, slower heating and cooling rates induced stiffer PPI gels and a linear relationship

between rheological parameters and heating and cooling rate was obtained. The minimum gelation concentration of PPIs with 10U MTG was 3% (w/v) compared to 5.5% that was reported previously for PPIs. At the same MTG level, the PPIs gel was stronger than that of SPIc gel, whereas when without MTG treatment, PPIs gel was weaker than that of SPIc gel. This indicates that PPIs with MTG treatment have the potential to substitute for SPIc as functional protein additives. SDS-PAGE was used to support the cross-linking of pea protein subunits as evidenced by fading or disappearances of high molecular weight bands (mainly at 74, 47, 41, 35 kDa), representing vicilin and the legumin acidic fractions.

Chapter 8: Gelation properties of myofibrillar/pea protein mixtures induced by transglutaminase crosslinking

8.1. Abstract

Gelation properties of myofibrillar protein isolate (MPI) and mixtures of MPI/pea protein isolate (PPI) were studied using a dynamic oscillatory rheometrer and a texture analyzer to evaluate PPI as a possible meat extender. For MPI, final heating temperature had a great impact on gel stiffness and the maximum gel stiffness was obtained at 95 ºC. pH and ionic strength also influenced gel stiffness and the maximum gel stiffness was achieved at pH 6, 0.9 M NaCl; however, good gels were formed in 0.6 and 1.2 M NaCl. In the MPI concentration range of ~ 0.5-5%, a positive correlation was observed between gel stiffness or gel peak force and MPI concentration. When MTG was included at levels of ~0 to 12-15 U, positive linear relations were found between gel stiffness or peak force and MTG levels. However, negative correlations for these parameters were observed at higher MTG concentrations. The inclusion of MTG increased the gel stiffness of MPI/PPI mixture (3% + 1%) more than it did for MPI (3%), but less than a 3%MPI + 1% soy protein isolate combination. Direct evidence of interaction between muscle and pea proteins in the form of new SDS-PAGE bands was not found; however, the improvement in gel stiffness or gel peak force for the MPI/PPI mixture (3% + 1%) with inclusion of MTG suggested that some ε (γ-glutamyl) lysine (G-L) crosslinking occurred, between muscle and pea proteins. It is likely that pea protein acted as a non-gelling component and interspersed throughout the primary MPI gel network and the addition of MTG promoted partial crosslinking between PPI and MPI. Consequently, MTG is useful in

improving gelation properties of heat-induced MPI/PPI gel and provides new opportunities to expand the utilization of pea protein in muscle foods.

8.2. Introduction

Consumer demand for low fat, high protein meat products has resulted in development of non-meat protein additives as fat or meat substitute. These non-meat protein additives aim at improving physical characteristics (e.g. texture-related properties), nutrition, flavor, and cooking yield, while reducing production cost. A number of non-meat proteins have been applied to ground or emulsified muscle foods as functional and nutritional ingredients, of which soy proteins are probably the most widely used (Pietrasik & Li-Chan, 2002). Due to the functionalities of soy proteins (isolates or concentrates), they are used in processed meats as binders to improve yield and texture, as emulsifiers, to enhance the emulsion stability upon heating, as gelling agents (Renkema & van Vliet, 2002), and to reduce the formulation costs (Chin et al., 1999).

Pea is widely grown in Canada and pea products such as pea protein isolate (PPI) are commercially available. Although PPI has been considered a non-meat protein additive and a substitute for soy protein isolate (SPI), limited literature was found on studying the gelation properties of comminuted muscle foods with addition of PPI (Su et al., 2000) or pea flour (Pietrasik & Janz, 2010). It has been demonstrated that gel stiffness of PPI is generally less than SPI (Su et al., 2000; Sun & Arntfield, 2010), therefore, it is expected that gel stiffness of comminuted muscle foods with addition of PPI will also be weaker than that of comminuted muscle foods with addition of SPI and this shortcoming should be addressed to improve the texture properties of meat products containing PPI.

Functional properties of pea protein isolates in comminuted meat products will depend, to a great extent, upon their interaction with muscle proteins and the formation of a continuous, cross-linked structure. It has been reported that soy proteins, including the two major globular fractions, β-conglycinin (7S) and glycinin (11S), are relatively resistant to denaturation (Feng & Xiong, 2002; Petruccelli & Añon, 1995b). Under the normal meat processing conditions (temperature 65-73 ºC, pH 5.5-6.0, and ionic strength 0.1-0.6), the major soy globulins do not undergo appreciable structural changes and consequently, interaction with muscle proteins is limited (Ramírez-Suárez & Xiong, 2003a). It was concluded that the lack of interaction between soy and meat proteins reduces the effectiveness of soy proteins as a functional component to improve gel stiffness and structure of comminuted and emulsified meats (Feng & Xiong, 2002; McCord et al., 1998). Pea proteins also consist of two major components (vicilin, 7S; legumin, 11S) which are very similar to those of soy proteins and do not denature at temperatures normally associated with meat processing (Shand et al., 2007). To use pea proteins in comminuted and emulsified meats, the lack of interaction between pea proteins and meat proteins is probably the major obstacle to producing strong gels.

When two or more proteins are mixed, there are several ways in which they can interact with each other and this is reflected in the properties of the gel formed. Incompatible, semicompatible or compatible states of the mixed proteins can be formed depending on the level of type of interactions between proteins (Manson & Sperling, 1976). Based on these interactions, five types of mixed gels have been reported (Ziegler & Foegeding, 1990). In an incompatible system, where one protein is present at a

concentration below its least gelation concentration, this protein may act as a filler or non-gelling agent within the network created by the other protein. Alternately, the two proteins may form independent networks as they are thermodynamically incompatible. These can be either interpenetrating continuous networks or localized networks for one protein within an overall network structure created by the other protein (Ziegler & Foegeding, 1990). Tolstoguzov (1986) has referred to the latter type of gel as a filled gel. In incompatible systems, the competition for solvent can allow the polymers to behave as though they were present at higher concentrations and gel independently. In a semicompatible system, a non gelling protein can associate with the network of the other protein and reduce the flexibility of the network and produce more rigid gels (Ziegler & Foegeding, 1990). If there is compatibility between two proteins or polymers, there can be either co-polymerization or coacervation where both polymers contribute to the network.

The overall objective is to effectively use PPI as an extender for gels formed by muscle protein. To achieve this, conditions needed to form MPI gels at temperatures higher than those normally used in meat production, will be investigated so that the PPI can be added at temperatures that will support denaturation of these proteins. In addition the effectiveness of a microbial transglutaminase (MTG) as a catalyst to promote interactions between proteins will be evaluated for the PPI/MPI mixed system and compared to the effect on MPI alone.

8.3. Materials and methods

8.3.1. Commercial pea flour and PPIs extraction procedure

Commercial pea flour was kindly donated by Best Cooking Pulse, Inc. (Portage la Prairie, MB, Canada). The salt-extracted pea protein isolate (PPIs) was extracted using the method of Sun & Arntfield (2010). PPIs after freeze drying (Genesis SQ Freeze Dryer, Gardiner, NY, U.S.A.) contained ~ 82% of protein as determined by Kjeldahl method using a N to protein conversion factor of 5.7 (AACC, 1982).

8.3.2. Commercial fine ground chicken breast and MPI extraction procedure

Commercial, finely ground chicken breast was purchased from a local grocery store and used immediately to extract myofibrillar protein. Myofibrillar protein isolate (MPI) preparation followed the procedure of Chen et al. (2003) with some modifications. Ground chicken breast (100 g) was homogenized in 400 mL double-distilled water for 1 min with a Waring blender at high speed. The suspension was then centrifuged at 2795×g for 20 min at 4ºC. The resulting pellet was resuspended in 400 mL of 0.1 M NaCl solution containing 50 mM Na_2HPO_4 and 1 mM NaN_3, at pH 6.0, and blended for 1 min at high speed. The suspension was then filtered with 4 layers of cheese cloth to remove connective tissue and centrifuged again at 2795×g for 20 min at 4 ºC. The homogenization (30 sec, high speed) and centrifugation steps were repeated four more times using 0.1 M NaCl solution (as described above) as washing buffer. The final MPI pellet was refrigerated and used within 1 week. The protein concentration in the MPI was determined by the Biuret method.

8.3.3. Sample preparation

Based on the protein content determined by biuret method, MPI was first diluted to desired concentrations using different concentrations of NaCl (0.1, 0.3 and 0.6 M) containing 50 mM Na_2HPO_4 at pH 6.0. Freeze-dried PPI was also diluted to the required concentrations using the same NaCl concentration solutions as described above. Then they were mixed together and homogenized for 30s to form MPI/PPI dispersions. For those samples to which MTG was added, the appropriate amount of MTG was added to the prepared slurry and incubated at 40ºC for a set time prior to futher analysis.

8.3.4. Rheology

MPI pellets were diluted to 0.5, 1.0, 1.7, 2.0, 2.7, 3.0, 3.5, 4.0, and 4.5% (w/v) using designed NaCl solution to achieve desired ionic strength. PPI dispersions were prepared as described above, pH was adjusted using 1 M NaCl or 1 M NaOH if necessary, and the samples were homogenized. For MPI/PPI mixtures adequate amounts of MPI and PPI were weighed and dispered in the desired NaCl solution to make 3% MPI/1% PPI, 2% MPI/2% PPI, and 1% MPI/3% PPI and then homogenized. Rheological parameters were determined using the method described by Sun & Arntfield (2010).

8.3.5. Texture profile analysis (TPA) of gels

For texture analysis, all samples were prepared as noted above in 50 mL beakers, heated to 95 ºC (to induce denaturation of pea proteins and allow for interaction between PPI and MPI) and kept at this temperature for 10 min in an oven, and then cooled down to room temperature overnight. A TA-XT2i texture analyzer equipped with a 1 cm diameter metal sphere probe was used to test gel strength of different samples in the beakers. The equipment was set as follow: pre-test speed: 4.0 mm/s; test speed: 0.1 mm/s;

post-test speed: 4.0 mm/s; rupture test distance: 1.0 mm; distance: 4.0 mm; force: 0.588 N; time: 0.1 sec; count: 5. As there was evidence of syneresis for some gels following gel formation, this liqud was removed from the surface prior to test. All samples were prepared in duplicate and tested 6 times. Gel peak force was formed at the end of deformation.

8.3.6. SDS-PAGE

SDS-PAGE was used to look at protein subunits of unprocessed MPI in the presence of MTG and in all the protein gels prepared. To evaluate the effect of various incubation times in the presence of MTG on G-L crosslinking of MPI at different salt concentrations, a 2% MPI dispersion (in 0.1, 0.3 and 0.6 M NaCl) with and without 10 U MTG was prepared and 0.5 mL were pipetted into 9 2-mL microcentrifuge tubes, and incubated in a water bath at 40 ºC for 10, 30, 60, 90, 120, 180, 240, 300, and 480 min. After incubation, 1 mL sample buffer was immediately added to the microcentrifuges tubes. Mixtures were vortexed for 1 min, boiled for 3 min, and vortexed for 1 additional min. These samples were ready for electrophoresis following the same procedure as was used for the protein gels.

To evaluate subunits in the protein gels the method of Aluko & McIntoch (2001) was followed with minor modifications. Gels used for TPA were removed from the beakers and mixed with 4 volumes of the same concentration NaCl solutions. These mixtures were blended using a blender at high speed for 1 min and from each dispersion 0.5 mL was pipetted into a microcentrifuge tube and 1 mL sample buffer (4% SDS (L-3771, Sigma, St.Louis, USA), 20% glycerol (Mallinckrodt Specialty Chemicals

Company, Paris, Kentucky, USA), 10% 2-mercaptoethanol (M-7154, Sigma, St. Louis, USA), 0.125 M Tris-HCl (T-1503, Sigma, St.Louis,USA), and 0.01% Pyronin Y (P-6653, Sigma, St. Louis, USA), pH 6.8) was added. Samples were vortexed (Vortx Genie 2, Scientific Industries Inc., Bohemia, USA) for 1 min, boiled for 3 min in a water bath and vortexed for 1 additional min to promote dissolution. The samples were then centrifuged at 2200 × g (Fisher Scientific, Cat. No. 05-090-128, Korea) to settle out any remaining particulate matter and 5µL of the supernatant were applied to each well of a gel containing a 4% acrylamide separating gel and 12% acrylamide separating gel. SDS-PAGE Molecular Weight Standards (Broad Range, Bio-Rad, Hercules, USA) were prepared as per manufacturers instructions and 8 µL were loaded into one well. Gels were run at 10 amp per gel (Mini-ProteanR 3 Cell, Boi-Rad, Hercules, USA) for about 3h until the dye reached the bottom of the gel. The gel was exposed to a staining solution consisting of 0.1% (w/v) Commassie Brilliant Blue G-250 (B-1131, Sigma, St. Louis, USA), 10% (w/v) acetic acid and 40% (w/v) methanol (fisher Scientific, Ottawa, Canada) for 3 h to visualize the protein bands. A destaining solution containing 20% methanol (ACS 093, BDH Inc., Toronto, Canada), 10% acetic acid was used to destain the gels. The gels were photographed using a Canon EOS 450D digital camera and a Bandscan software 5.0 was used to analyze band intensity and calculate molecular weights.

8.3.7. Differential scanning calorimetry

The thermal properties of the MPI and MPI/PPI suspensions were examined using a DSC Q200 (TA Instruments, New Castle, DE, USA). Instrumental conditions were as described previously (Sun & Arntfield, 2010). Peak transition temperature or denaturation temperature (T_d), and enthalpy of denaturation (ΔH) were computed from

the endothermic peaks observed in the thermograms. Each sample was analysed in duplicate.

8.3.8. Statistical analysis

All data were analysed by one way analysis of variance (ANOVA) for significant differences, with minimum significance set at the 5% level ($P<0.05$), followed by Tukey's test to find differences, GraphPad InStat software version 3.06 (GraphPad Software Inc. La Jolla, CA, USA) was used for the statistical analysis.

8.4. Results and discussion

To investigate the impact of PPI inclusion on the gelation of chicken MPI, an initial investigation of MPI gelling properties was conducted prior to looking at the MPI/PPI mixtures.

8.4.1. Impact of various factors on gelation properties of MPI

8.4.1.1. Impact of heating temperatures on structural and rheological properties of MPI

As heating temperatures used in the gelation of animal and plant proteins are very different, the impact of heating temperature on the gelation of MPI was investigated. As seen in Fig. 8.1, there is no significant difference in G' between final heating temperatures 45 and 55 ºC; however, significant differences were found among final heating temperatures 65, 75, 85 and 95 ºC with higher G' values for higher temperatures. For tan δ, no significant difference was found among the values at final heating temperatures 55, 65, 75, 85 and 95 ºC; only the value at final heating temperature 45 ºC

was significantly higher than the others. This indicated that different final heating temperatures greatly influenced the rheological properties of MPI. Myosin has been reported to denature at about 65 ºC (Feng & Xiong, 2002, 2003). This would explain the significant increase in G' from 55 ºC to 65 ºC, because when the temperature exceeds the myosin denaturation temperature, molecules unfold and expose their active groups enabling cross-linking and enhanced gel stiffness. As G' values reached the maximum at 95 ºC, this indicated that more active groups were exposed and gel stiffness was at its highest.

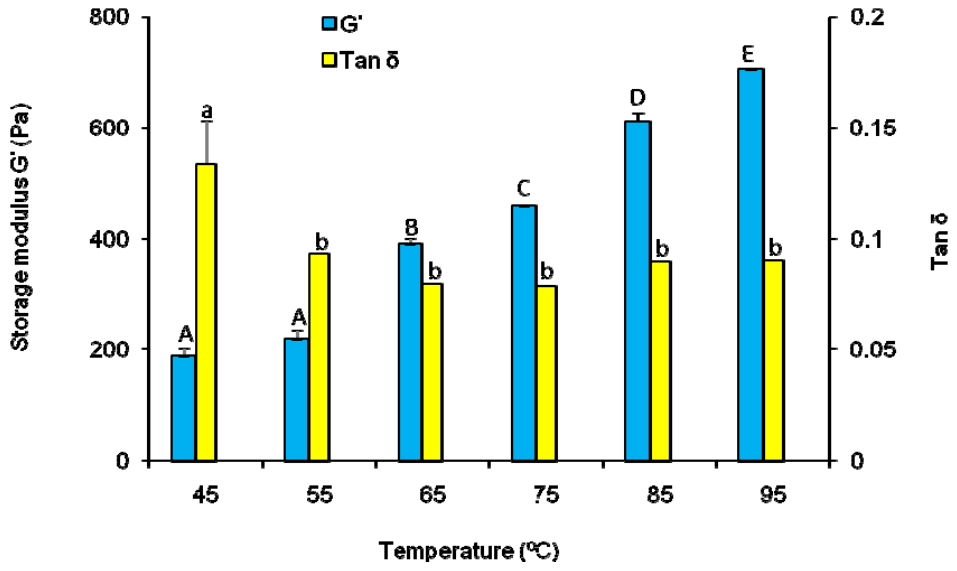

Fig. 8.1 Impact of final heating temperatures on rheological properties of MPI (2% MPI (w/v) in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.0).

As seen in Fig. 8.2, the myosin heavy chain (MHC) band disappeared in all the treatments regardless of final temperatures, whereas the actin band intensity gradually increased with increasing final heating temperature. This is probably an indication of

aggregates or crosslinks formation involving MHC molecules such that the larger polymers formed could not enter the separating gel. In the study of Feng & Xiong (2002), they observed that at 60 ºC both pork MHC and actin diminished abruptly and they ascribed this phenomenon to proteins insolubilization resulting from their denaturation. Their observation was different from our result; as in our experiment MHC disappeared at 45 ºC the actin band intensity tended to increase rather than abruptly diminished at 60 ºC. It is possible that the difference in meat protein sources was responsible as the MPI we employed was extracted from chicken breast while that used by Feng & Xiong was extracted from pork steak.

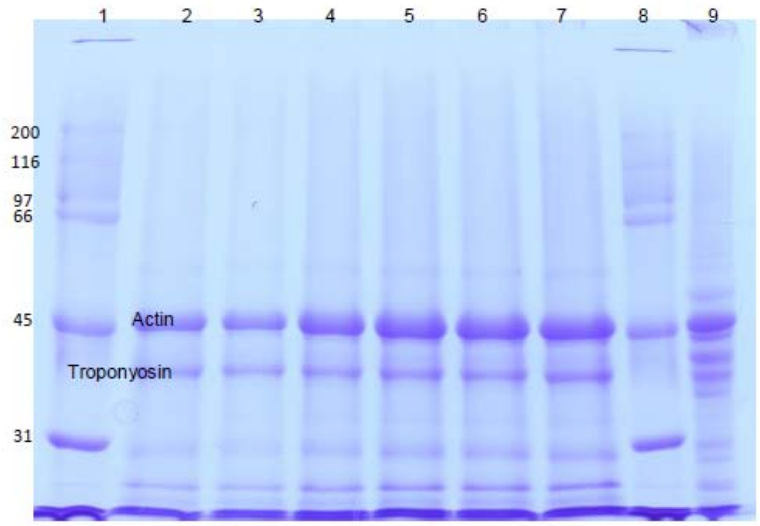

Fig. 8.2 Electrophoretic patterns of myofibrillar protein isolate (MPI, 2%, w/v) in 0.6M NaCl buffer heated to various temperatures during gelation. Lane 1: MW marker, Lane 2: 45 ºC, Lane 3: 55 ºC, Lane 4: 65 ºC, Lane 5: 75 ºC, Lane 6: 85 ºC, Lane 7: 95 ºC, Lane 8: MW marker, Lane 9: pure chicken breast heated to 95 ºC.

8.4.1.2. Impact of NaCl concentration on rheological properties of MPI

NaCl also had a significant impact on gelation properties of MPI. G' achieved the greatest value at high salt concentration of 0.9 M NaCl. At 0.1 and 0.3 M NaCl, G' values were the smallest and not significantly different (Fig. 8.3). Gels could not form at these low salt levels. The tan δ value was the greatest at 0.3 M NaCl; no significant differences in tan δ were seen at other salt levels. This suggests that although MPI maximum gel stiffness occurred at 0.9 M NaCl, strong well crosslinked gels were formed in 0.6 M and 1.2 M NaCl. Weak and relatively non elastic networks were obtained with 0.1 and 0.3 M NaCl, respectively.

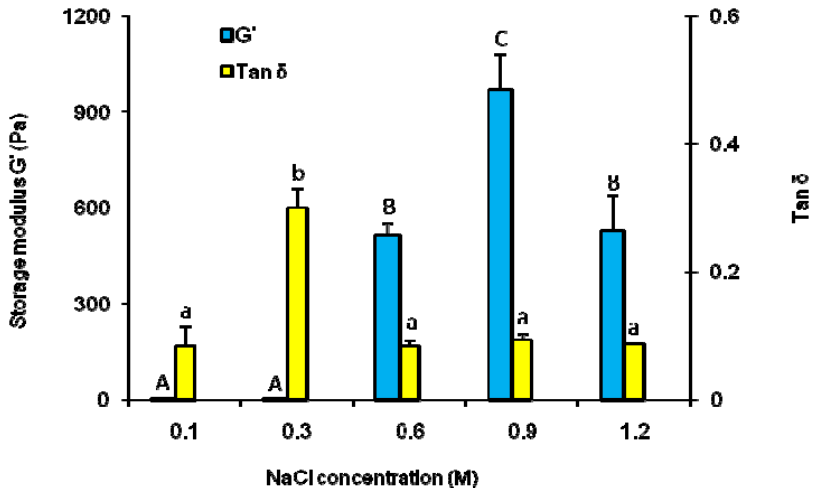

Fig. 8.3 Impact of NaCl concentration on rheological properties of MPI (2% MPI (w/v) in 50 mM Na_2HPO_4 at pH 6.0).

Due to health concerns related to high salt intake which can cause heart disease and high blood pressure (Health Canada, 2010), there is increased pressure to reduce salt intake. For meat products such as sausage, usually salt content is in the range of 0.5~4%,

equivalent to about 0.1~0.7 M. Although at 0.9 M NaCl, chicken MPI forms the strongest gel (Fig. 8.3), this level is higher than what is desired for consumers. MPI can form good gel at 0.6 M NaCl (equivalent to 3.5%), and although this is still a relatively high salt concentration it is in the range currently used. Any further salt reduction was at the cost of lost MPI gel strength.

8.4.1.3. Impact of pH on rheological properties of MPI

As seen in Fig. 8.4, pH has a significant influence on gelation properties of MPI. At pH 6 and 7, G' values were significantly greater than at other pH values, and G' was significantly higher at pH 6 than at pH 7, which means MPI formed a stronger gel at pH 6 than at pH 7. Ishioroshi et al. (1979) indicated that the optimal pH for heat-induced gelation of myosin was 6.0. Xiong & Brekke (1991) indicated that the optimum pH for gelation of chicken muscle in 0.6 M NaCl or KCl was about 6.0 for breast myofibrils. Lesiów & Xiong (2003) found that the strongest gel from chicken breast muscle homogenates was formed at pH of 6.3. Our result is generally in agreement with the above observations. The maximum tan δ values were obtained at pH 4, 5 and 8 and no statistical difference were found among them. No significant differences in tan δ values were observed among pH 3, 6, 7, 9, and 10. However, tan δ values of these two groups were significantly different ($p<0.01$). This indicated that when MPI gel stiffness was the strongest at pH 6 and 7, the relative elasticity (low tan δ) of MPI gels remained high. Although gel stiffness at pH 4, 5, and 8, was not significantly different from that at pH 3, 9, and 10, the significant differences in relative elasticity that existed between these two groups indicated gels in the first groups had less crosslinking and were less desirable.

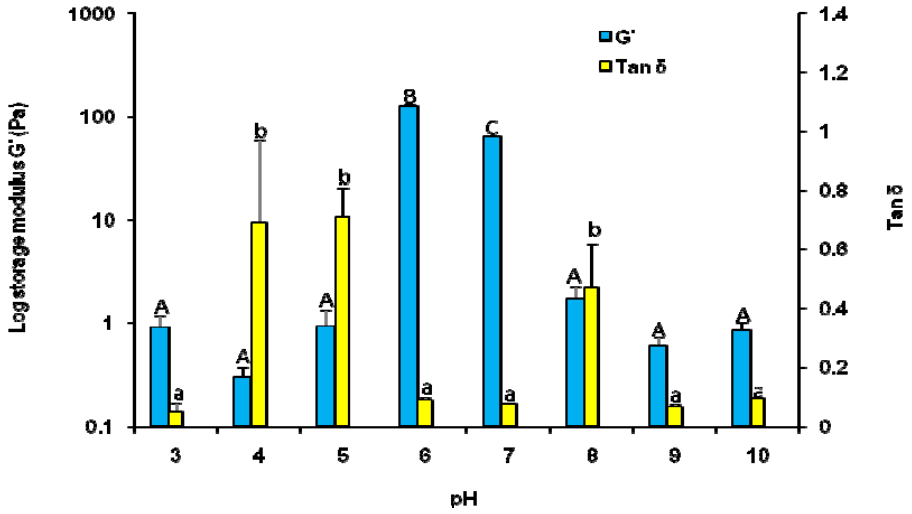

Fig. 8.4 Impact of pH on rheological properties of MPI (1% MPI (w/v) in 0.6 M NaCl, 50 mM Na_2HPO_4).

8.4.1.4. Impact of protein concentrations on gelation properties of MPI

Protein concentrations have great impact on their gelation properties. Power law relationships were obtained for both G' and MPI concentration and G" and MPI concentration (Fig. 8.5). Tan δ values exhibited minimal change over this concentration range.

Although a power law relationship between protein concentration and G' was obtained with dynamic rheological testing, a linear relationship was more appropriate when describing the relationship between protein concentration and gel peak force determined by texture analyzer (large deformation test) (Fig. 8.6). This is because the testing mechanisms are completely different. The rheometer monitors dynamic changes during gel formation, while texture analyzer deforms the end product. Liu & Xiong

(1997) indicated that the gel strength data obtained in gel penetration measurements were not necessarily consistent with the findings of the final G' in dynamic rheological tests. A gel penetration test is destructive, while a dynamic rheological measurement is nondestructive. They concluded that these two tests represent different aspects of the rheological profile of protein gels, consequently, may not be related.

In general, the two measurements indicated that both gel strength and gel stiffness increase with increasing protein concentration.

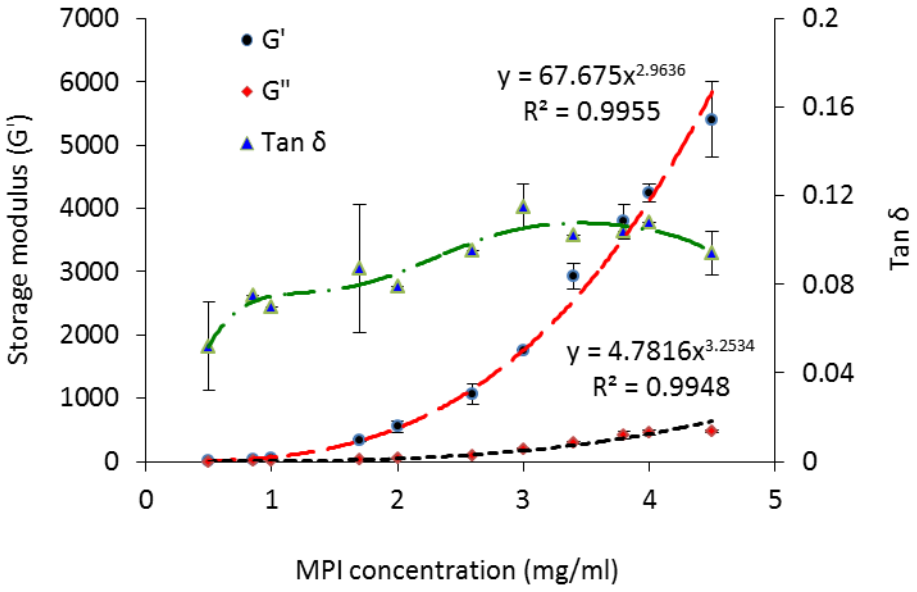

Fig. 8.5 Impact of MPI concentrations on its rheological properties (MPI dispersed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.0).

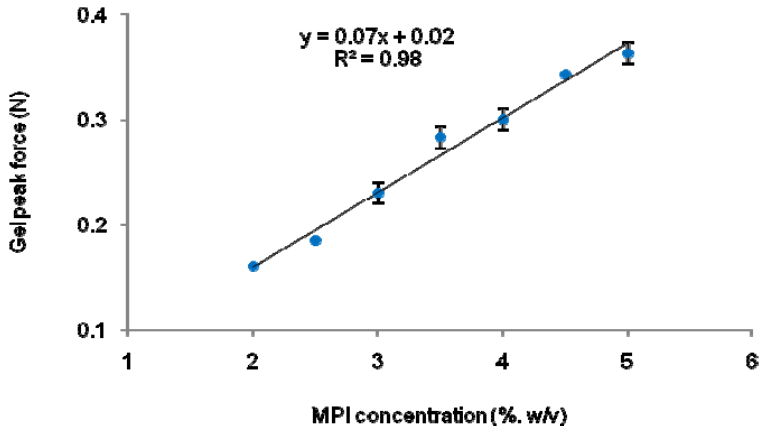

Fig. 8.6 Impact of MPI concentration on peak force of heat induced gel. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

8.4.2. Impact of MPI/PPI combinations on gelation properties of mixed protein gels

To better understand the changes occurring during gel formation, subunits were evaluated in gels formed under various conditions. To identify the protein components which were involved in the gel network formation under certain circumstances, electrophoresis was conducted to establish the role of different factors in gelation process.

8.4.2.1. Impact of MPI/PPI combinations on rheological properties

Rheological properties of MPI/PPI mixtures were evaluated using dynamic oscillatory rheology and the results from the final evaluation of the gels formed are shown in Fig. 8.7. The overall observation was that 4% and 3% MPI could form good gels (high G' and low tan δ values) whereas 4% PPI could not form a gel (low G' and high tan δ values). Incorpation of PPI to MPI decreased gel stiffness of MPI. It can be

seen that G' value of 3% MPI + 1% PPI (834 Pa) was significantly lower than 4% MPI (4241 Pa), and 3% MPI (1758 Pa) alone, which indicates that PPI does not crosslink with MPI and, thus does not have any positive effect on the gelation of MPI. Similar findings were reported by Ramírez-Suárez & Xiong (2002; 2003b) using other nonmuscle additives. Ramírez-Suárez & Xiong (2003a) indicated that untreated exogenous proteins had a detrimental effect on muscle protein gelation due to interference with the protein-protein interaction responsible for the formation of the elastic structure. In this case pea protein probably interfered with protein-protein interaction of myofibrillar molecules. Similar results were also presented by McCord et al. (1998) and Feng & Xiong (2002, 2003), who also observed decreased gel strength with addition of non-meat soy proteins. They attributed the decrease in gel strength to the presence of β-conglycinin (7S) soy protein which reduced self aggregation of myosin heavy chain during heating. Since pea proteins have been shown to exhibit comparable and complementary functional properties to soy proteins (O'Kane et al., 2005; Soral-Śmietana et al., 1998; Sosulski et al., 1976), it is possible that the pea vicilin diminished self aggregation of myosin heavy chains during heating and consequently resulted in the gel weakening effect. With decreasing MPI concentration (from 4 to 1%), the storage modulus of the gels decreased significantly and there was no difference between 1% MPI + 3% PPI and 4% PPI. It can be concluded that the addition of PPI has a negative effect on MPI gelation.

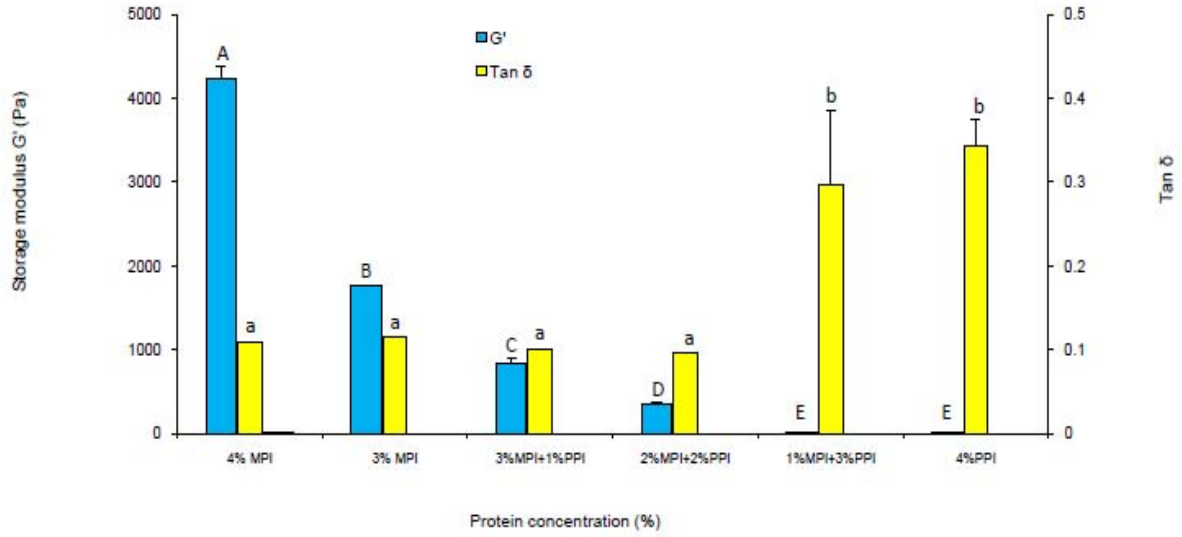

Fig. 8.7 Impact of MPI/PPI protein combinations on rheological properties of mixed protein gels. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

Based on the above results, it is possible that an incompatible state of the mixed proteins was achieved. According to Ziegler & Foegeding (1990), when two proteins which can both form gels are mixed and the concentration of one is below its least gelation concentration whereas the second is above its least gelation concentration, the second protein will form a gel while the first may act as a non-gelling filler interspersed throughout the gel network (Ziegler & Foegeding, 1990). This is likely the case for the PPI-MPI combination. As previously indicated, the least gelation concentration of PPIs is 5.5%, whereas MPI can form a gel at concentration as low as 0.5% (Arntfield et al., 1990). Therefore, it is likely MPI formed a gel network while PPI remained its liquid state and acted as a filler. This resulted in decreased gel strength. However, according to Ziegler & Foegeding (1990), PPI is not an inactive filler because it somewhat interfered with the MPI gel network formation.

8.4.2.2. Effect of combining MPI and PPI on the electrophoretic pattens of mixed protein gels

Electrophoretic patterns of MPI/PPI mixtures heated to 95 ºC for 10 min in 0.1, 0.3, and 0.6 M NaCl buffer were essentially identical (Fig. 8.8 a, b, c). With decreasing MPI to PPI ratios, band intensities of MHC, actin, tropomyosin, and troponin T gradually decreased. Conversely, with increasing PPI to MPI ratios, band intensities of pea vicilin proteins (V), pea legumin acidic subunit (L_α), and pea legumin basic subunit (L_β) gradually increased. No new bands were found in the MPI/PPI mixtures and this is different from the findings reported for a MPI/SPI combination (Ramírez-Suárez & Xiong, 2003a).

8.4.2.3. Changes in thermal behavior for proteins in mixed system

The effect of MPI/PPI ratio on thermal data is shown in Table 8.1. The 4% PPI sample had two endothermic transitions (~ 94 ºC and ~ 108 ºC) corresponding to the two major proteins in pea protein isolate (legumin and vicilin), while the 4% MPI sample had only one endothermic transition (74.5 ºC) corresponding to the major component (actin) in MPI. Feng & Xiong (2003) observed two transitions for pure MPI, 65.3 ºC and 74.7 ºC corresponding to pork myosin and actin, respectively. However, no transition corresponding to chicken myosin was observed for pure MPI. In addition, no transition was observed for actin when MPI was mixed with PPI. With increasing PPI ratio, the denaturation temperatures of two major components of PPI remained constant, while the enthalpies of the two components (ΔH_1 and ΔH_2) gradually increased which represents increased interactions between PPI proteins at higher ratios.

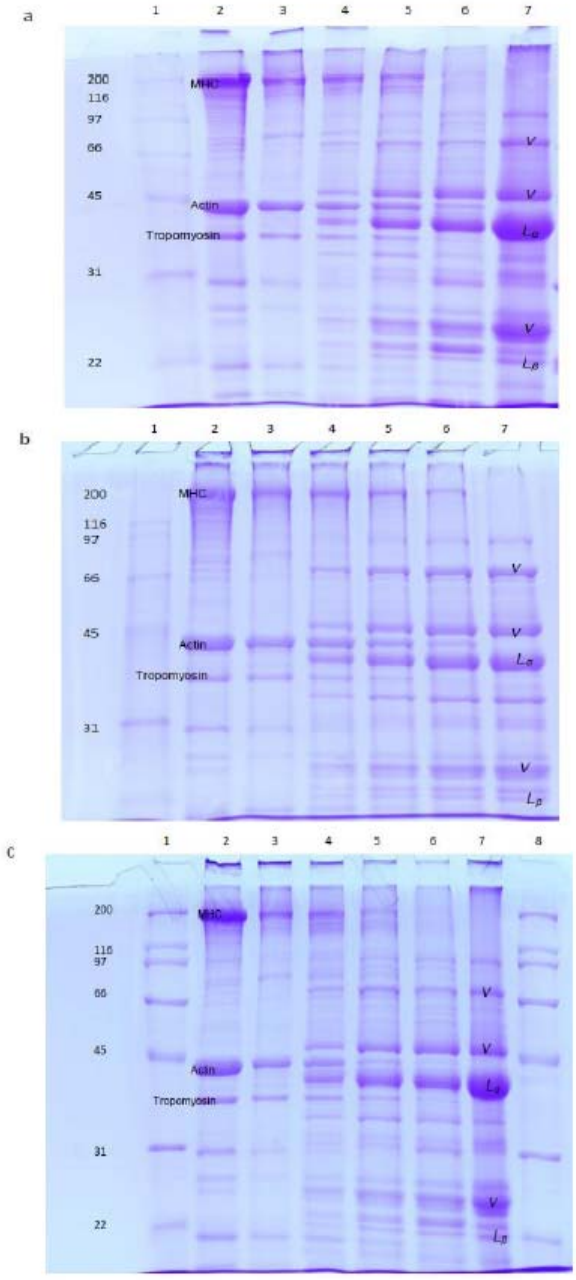

Fig. 8.8 Electrophoretic patterns of myofibrillar/pea protein isolate mixture heated to 95 °C for 10 min to form gels in 0.1 M (a), 0.3M (b), and 0.6 M (c) NaCl buffer. Lane 1: MW standard marker, Lane 2: 1% MPI (original, not heated to 95 °C to form gel), Lane 3: 4% MPI, Lane 4: 3% MPI + 1% PPI, Lane 5: 2% MPI + 2% PPI, Lane 6: 1% MPI + 3% PPI, Lane 7: 4% PPI, Lane 8: MW standard marker. *V* represents pea vicilin proteins, L_α represents pea legumin acidic subunit, and L_β represents pea legumin basic subunit.

Table 8.1 DSC data of various combinations of MPI/PPI in 0.6 M NaCl buffer

Combinations	T_{d1} (ºC)	T_{d2} (ºC)	ΔH_1 (J/g)	ΔH_2 (J/g)
4% MPI	74.5		0.49	
3% MPI/1% PPI	94.0	108.6	0.20	0.66
2% MPI/2% PPI	94.2	106.1	0.65	1.48
1% MPI/3% PPI	94.8	107.0	0.98	2.31
4% PPI	94.1	108.7	1.71	4.11

8.4.3. Impact of microbial transglutaminase (MTG) on the gelation properties of MPI and MPI/PPI mixtures

As MTG catalyzes crosslinks between glutamine and lysine, it is expected that gel stiffness of MPI and MPI/PPI mixtures can be increased. To better understand the impact on the mixed systems, it is important to know how MTG affects MPI without other proteins added.

8.4.3.1. Impact of MTG levels on gelation properties of MPI gels

The effects of MTG on the rheological properties of MPI during heating and cooling are shown in Figs. 8.9 and 8.10, respectively. The rheograms obtained from duplicates of different MTG levels are essentially identical and therefore, only one set of data is presented. No crossover point between storage modulus (G') and loss modulus (G", not shown in the figure) was observed in the heating phase, indicating that network structures formed in the samples prior to rheological testing.

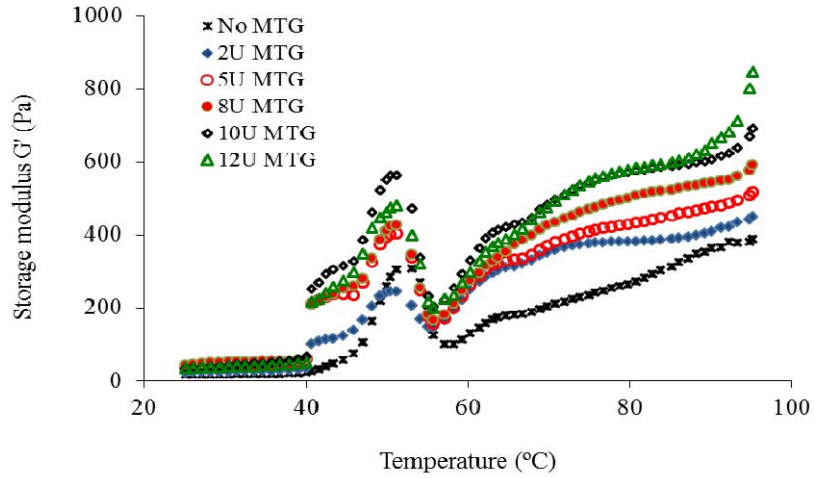

Fig. 8.9 Representative rheograms of G' values during heating of heat-induced MPI (2% protein concentration) gels with or without MTG during heating from 25 to 95 ºC at a rate of 2 ºC/min. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

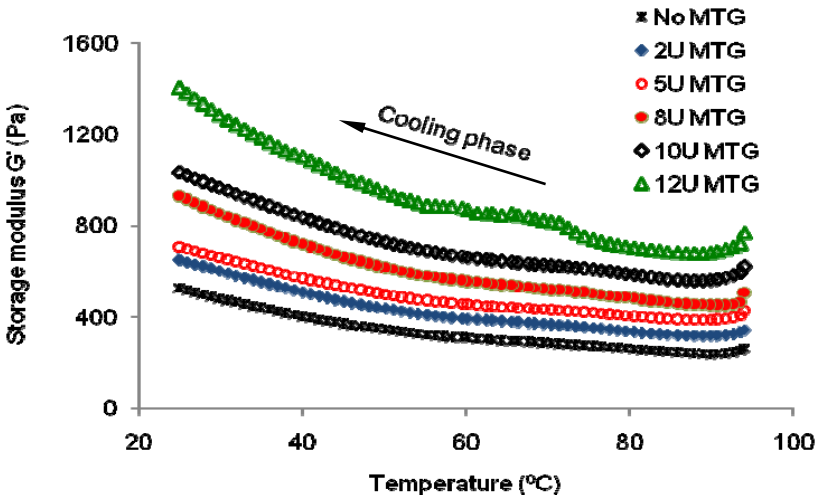

Fig. 8.10 Representative rheograms of G' values during cooling of heat-induced MPI (2% w/v protein concentration) gels with or without MTG during cooling from 95 to 25 ºC at a rate of 2 ºC/min. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

During the initial part of the heating stage, all samples showed a slow and steady increase in G' values. The gaps observed at 40 ºC for all the curves except the one without MTG treatments (control), is because there was a 30 min incubation period and during this period, enzyme-catalyzed crosslinking occurred between Glu and Lys residues which resulted in increased G' values when heating resumed. The G' curves peaked at about 50~52 ºC for all samples indicating that there was a sudden increase in gel stiffness followed by a rapid drop. This behavior has been previously investigated. Fernández et al. (1996) indicated that the first transition at ~ 48.5 ºC was likely caused by denaturation and aggregation of the myosin heads. Egelandsdal et al. (1986) suggested that denaturation of heavy meromyosin, and crosslinking of myosin filaments were responsible for the initial G' increase at < 50°C and denaturation of light meromyosin and increased filamental "fluidity", caused G' to decrease at temperatures > 50 ºC. They also speculated that the formation of more permanent, irreversible myosin filaments or complexes resulted in the G' increase thereafter. Other researchers suggested that the presence of actin filaments was responsible for the initial steep rise in rigidity (Boyer et al., 1996).

The maximum value of G′ (245.6 Pa) at ~ 51 ºC for the 2 U MTG treatment was slightly lower than the G' value at ~ 52 ºC for no MTG (control, 307.5 Pa), probably because the heating at temperatures < 50 ºC MTG had already catalyzed crosslinking of myofibrillar proteins, and the more compact structure formed limited further gel network formation (Oakenfull et al., 1997).

For all treatments containing MTG levels (2, 5, 8, 10, and 12 U), the maximum values for G' occurred at ~ 51 ºC, a temperature slightly lower than that seen for the control (~52 ºC). This was probably due to formation of more crosslinks at lower temperatures. The second increase in G′ for MTG treated samples was observed to start at ~ 56 ºC compared to ~ 58 ºC for control, and the slope was steeper in the 60 ~ 70 ºC range. This probably means MTG helped produce more intra- and intermolecular crosslinks and these crosslinked proteins had a lower temperature requirement for producing an elastic structure (Ramírez-Suárez & Xiong, 2003a). At temperature above ~ 75 ºC, the slope became less steep and this was probably caused by high temperature denaturation of MTG, which ended its ability to catalyze crosslinks. It can be noted that the maximum value of G' for 10 U MTG (564 Pa) was higher than for 12 U MTG (480 Pa). This indicated that 12 U MTG level was slightly higher than the optimum enzyme and excess enzyme can adversely affect gelation. However, G' values of 12 U MTG treatment were greater than for the 10 U MTG sample at higher temperature (> 85 ºC). Rather than enzyme catalyzed crosslinking, it is possible that hydrophobic interactions were involved and MTG was also involved in these interactions. At the end of the heating phase (95 ºC), 12 U MTG treatment had achieved the greatest G' value, followed by 10 U, 8 U, 5 U, and 2 U, and the MTG free control.

During the cooling phase (Fig. 8.10), the gel stiffness of all samples gradually increased and samples with higher MTG levels had greater gel stiffness. Apparently, MTG has a great impact on gel stiffness of MPI and higher levels MTG can greatly enhance gel stiffness.

The impact of MTG on tan δ is shown in Figs. 8. 11 and 8.12. Fig. 8.11 shows the heating phase of MPI with various MTG levels. Without MTG (control), tan δ peaked at about 45 ºC. In contrast, all the samples with MTG, tan δ values dropped significantly following incubation at 40 ºC. The increase in tan δ values in the range of 45 ~ 55 ºC, corresponds to the sharp rise in G' in the same temperatures (Fig. 8.9). This can be explained by the reaction of actin filaments (Boyer et al., 1996) and a more fluid system. With increasing temperatures, tan δ values gradually decreased to very low levels as protein interactions resulted in increased cross-linking. In general, the tan δ curves with or without MTG entangled in the temperature range of 70 ~ 95 ºC, and no differences in elasticity (tan δ) were seen in this temperature range.

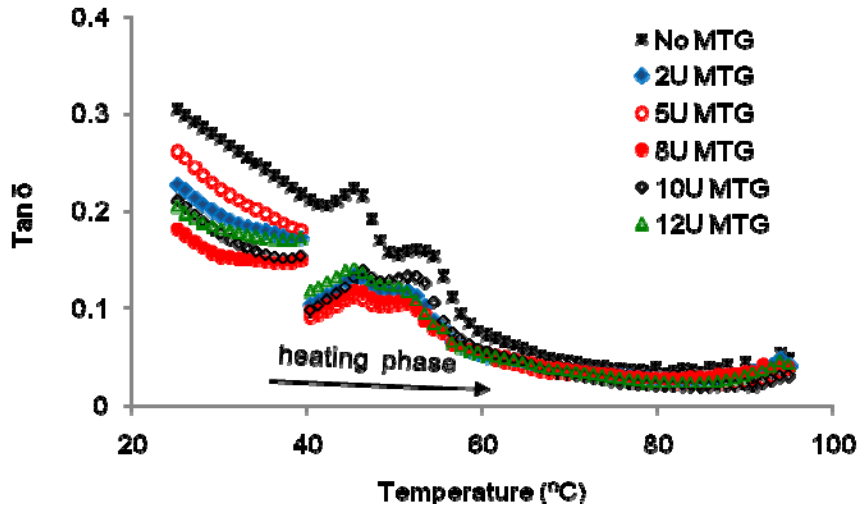

Fig. 8.11 Representative rheograms of tan δ values during heating of heat-induced MPI (2% protein concentration) gels with different MTG levels during heating from 25 to 95 ºC at a rate of 2 ºC/min. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

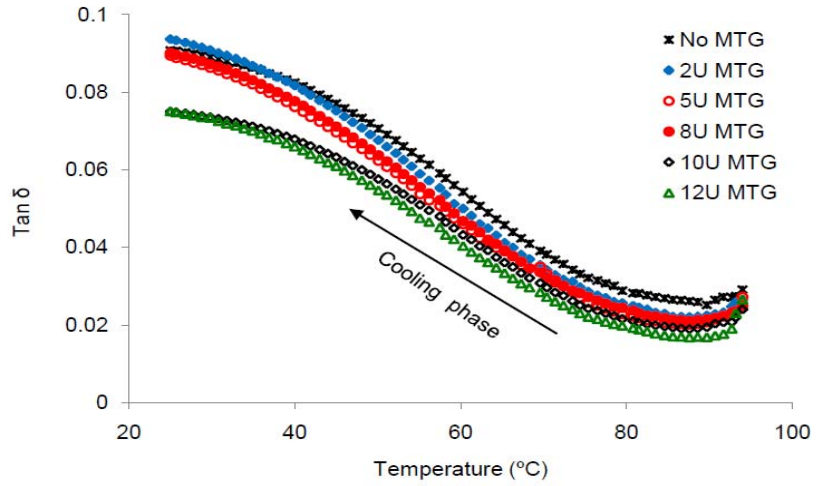

Fig. 8.12 Representative rheograms of tan δ values during cooling of heat-induced MPI (2% w/v protein concentration) gels with different MTG levels during cooling from 95 to 25 ºC at a rate of 2 ºC/min. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

Changes in tan δ during the cooling phase are shown in Fig. 8.12. It can be seen that tan δ values remain rather stable in the temperature range of 95 ~ 80 ºC, and with further cooling, values increased. This means the elasticity was reduced during the cooling stage. It can be seen that higher levels of MTG induced lower tan δ values. MTG levels of 10 and 12 U resulted in lower tan δ values than at lower MTG levels.

The impact of MTG on G' and tan δ over an extended range of MTG concentrations is summarized in Fig. 8.13. It could be seen that MTG level of 12 U/g protein was a turning point for G' values. At MTG levels lower than 12 U, G' values increased with increasing MTG levels; however, when MTG levels were higher than 12 U, G' values decreased with increasing MTG levels. A linear relationship was observed between MTG levels and G' values in the MTG level range of 0 ~ 12 U, as shown in the

inset in the top right corner. Tan δ values fluctuated around 0.08 with the lowest tan δ occurring at 10 U MTG which indicated that elasticity was maximum at this point.

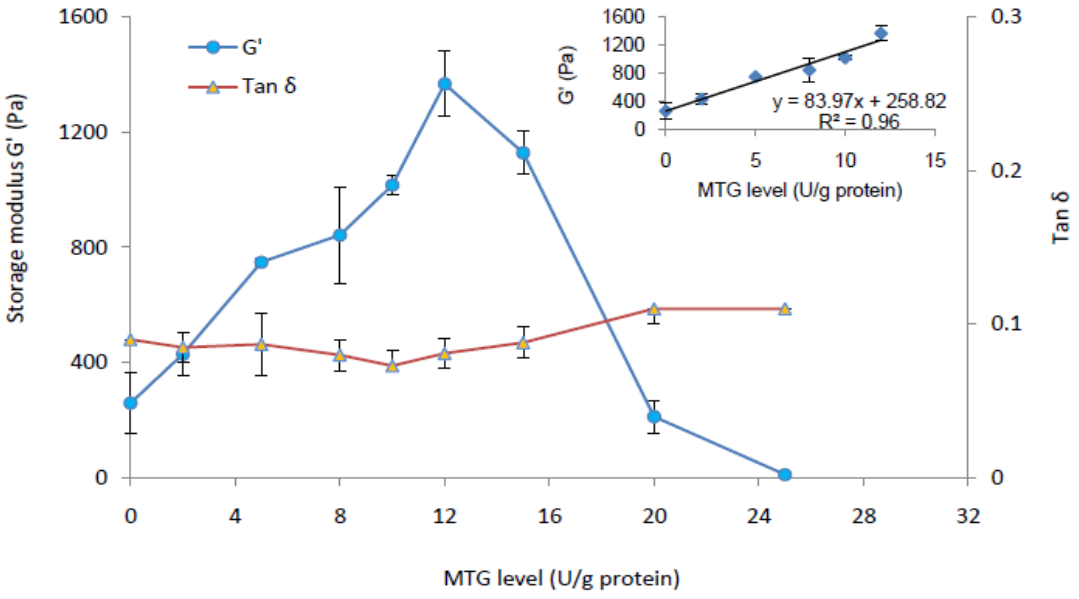

Fig. 8.13 Impact of MTG level on rheological properties of heat induced MPI gel. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6. Enlarged MTG level scale region (0 ~ 12 U/g protein) for G' in the top right corner represents a linear regression with MTG level.

The gel strength of MPI gels prepared with various MTG levels was also assessed by measuring the penetration force for the pre-formed gels (Fig. 8.14). The response was similar to that seen for G' with peak force increasing at MTG level between 2~15 U and, then decreasing at MTG levels between 15~25 U. In MTG level range of 2 ~ 15 U (Fig. 8.14), the following equation was able to describe the relationship between peak force and MTG concentration:

$$y = 0.02\ x + 0.45,\ R^2 = 0.98$$

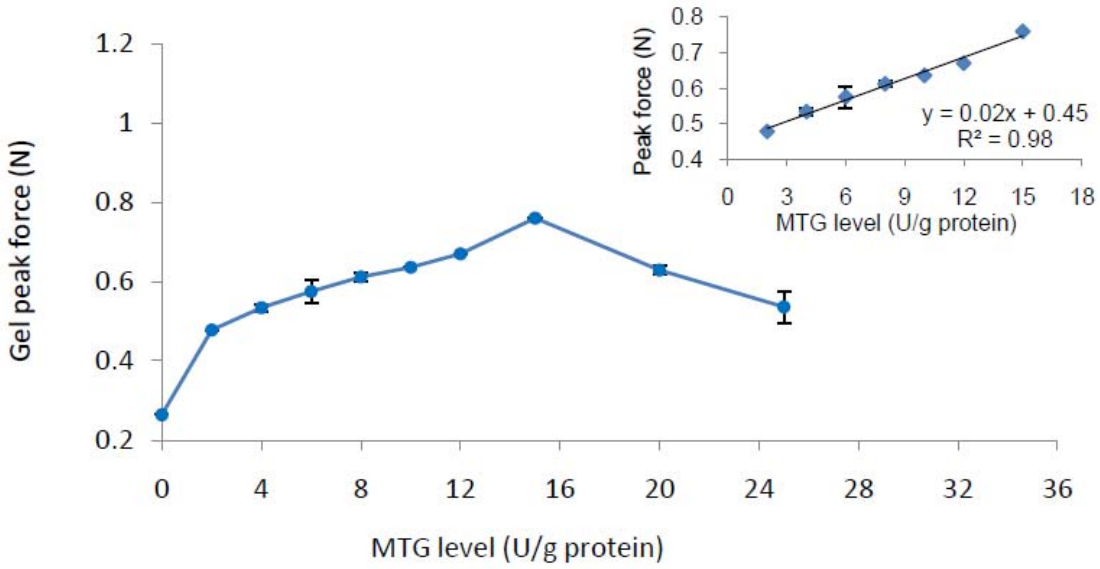

Fig. 8.14 Impact of MTG level on peak force of heat induced MPI gel. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6. Enlarged MTG level scale region (0 ~ 15 U) for gel peak force in the top right corner represents a linear regression with MTG levels.

8.4.3.2. Effect of MTG addition on the electrophoresis and thermal behavior of MPI

To investigate the role of MTG on the protein subunits involved in the gelation of MPI, 10 U/g protein of MTG was applied to MPI at different salt levels for various incubation times. Electrophoretic patterns of the myofibrillar protein isolate incubated at 40 ºC with 10 U /g protein MTG for different times in 0.1, 0.3, and 0.6 M NaCl buffer are shown in Fig. 8.15 a, b, and c, respectively. In general, the band intensity of major myofibrillar protein component myosin heavy chain (MHC), actin, and tropomyosin gradually decreased and even disappeared as incubation time increased.

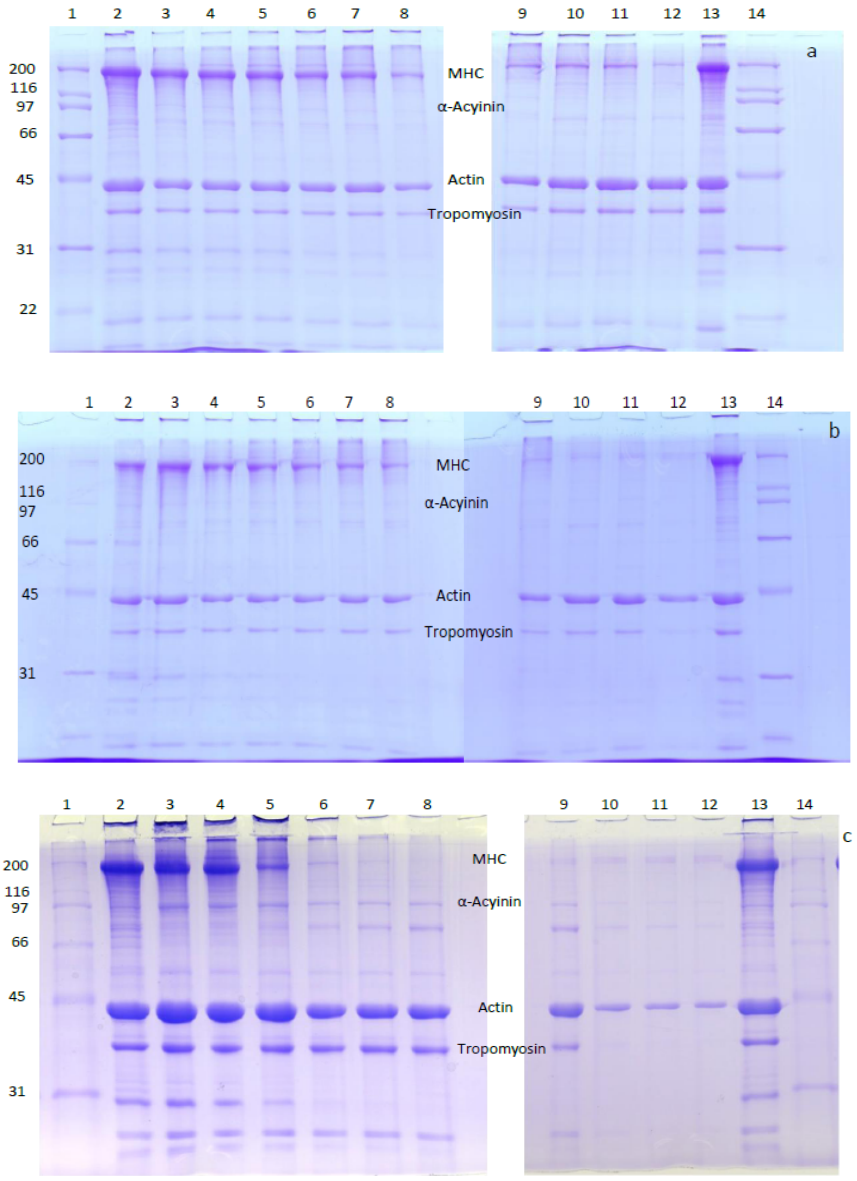

Fig. 8.15 Electrophoretic patterns of myofibrillar protein isolate (MPI, 1%, w/v) incubated at 40 ºC with 10 U MTG for different times in 0.1M (a), 0.3 M (b), and 0.6 M (c) NaCl buffer. No heat-induced gelation. Lane 1: MW standard marker, Lane 2: 1% MPI, no MTG, Lane 3: 1% MPI, 10 U MTG, 0 min, Lane 4: 1% MPI, 10 U MTG, 10 min, Lane 5: 1% MPI, 10 U MTG, 30 min, Lane 6: 1% MPI, 10 U MTG, 1 h, Lane 7: 1% MPI, 10 U MTG, 1.5 h, Lane 8: 1% MPI, 10 U MTG, 2 h, Lane 9: 1% MPI, 10 U MTG, 3 h, Lane 10: 1% MPI, 10 U MTG, 4 h, Lane11: 1% MPI, 10 U MTG, 5 h, Lane 12: 1% MPI, 10 U MTG, 8 h, Lane 13: 1% MPI, no MTG, Lane 14: MW standard marker.

For MHC, band intensity decreased slowly in 0.1 M NaCl buffer and was still present after an 8h incubation time. In 0.3 M NaCl buffer, MHC band disappeared more quickly and almost disappeared at 9h. The fastest disappearance of MHC occurred in 0.6 M NaCl buffer at ~ 6h. For actin, there was an increase in band intensity with increasing incubation time in 0.1 M NaCl buffer while in 0.3 M NaCl buffer, actin band intensity remained constant. In 0.6 M NaCl buffer, band intensity decreased as incubation time increased. A very similar response was also found for tropomyosin. It is most likely that with MTG catalyzed crosslinking at higher salt concentration, MHC, actin, and tropomyosin can form intra- or intermolecular crosslinked high molecular weight aggregates or polymers that were too big to enter the separating gel. At 0.1 M the aggregates may be found with MHC alone. Actin was resistant to enzyme catalyzed crosslinking over a broad range of incubation time in all three NaCl concentration levels and this is in agreement with the finding of Ramírez-Suárez & Xiong (2002).

Fig. 8.16 shows electrophoretic patterns of 2% MPI treated with various levels of MTG in 0.6 M NaCl buffer for 30 min and then heated to form a gel. It is not surprising that the MHC band in all treatments disappeared, as the MHC was previously shown (Fig. 8.2) to form aggregates that could not enter the separating gel and, therefore, did not appear as a band. The intensity of actin band did not change between treatments indicating it is not involved in enzyme catalyzed crosslinking (Ramírez-Suárez & Xiong, 2002).

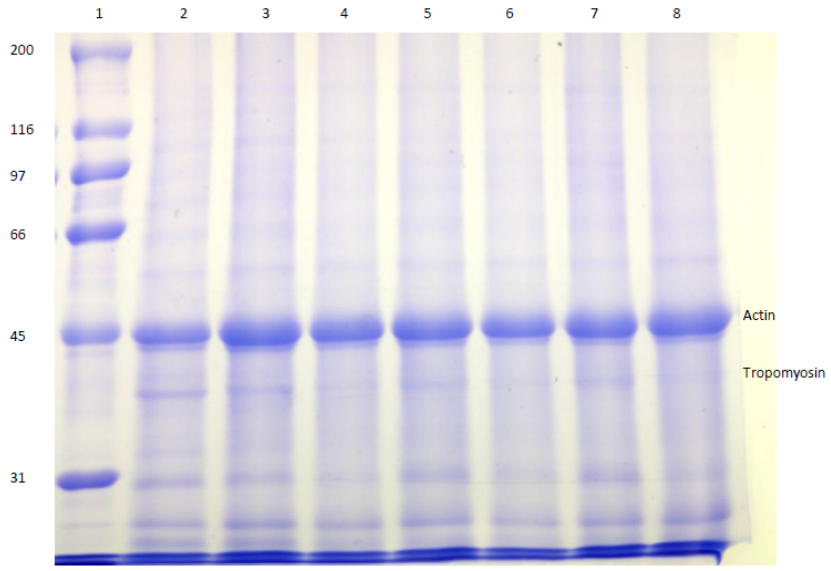

Fig. 8.16 Electrophoretic patterns of 2% (w/v) MPI with various amount of MTG incubated at 40 ºC for 30 min in 0.6 NaCl buffer, and then heated to 95 ºC to form gels. Lane 1: MW marker, Lane 2: 2% MPI, Lane 3: 2% MPI, 2 U MTG, Lane 4: 2% MPI, 5 U MTG, Lane 5: 2% MPI, 8 U MTG, Lane 6: 2% MPI, 10 U MTG, Lane 7: 2% MPI, 12 U MTG, Lane 8: 2% MPI, 15 U MTG.

8.4.3.3. Impact of adding MTG on gelation properties of MPI/PPI mixtures

Rheological properties of MPI/PPI mixtures containing 10 U/g protein MTG in 0.6 M NaCl are shown in Fig. 8.17 and the same trend was found as was seen without adding MTG. There were significant differences among the samples. Although gel stiffness increased with addition of MTG, the G' value of 3% MPI + 1% PPI was smaller than 3% MPI indicating that there was still a lack of interaction between MPI and PPI molecules. The increases in G' from adding MTG were probably caused by MTG induced cross-linking between glutamine and lysine residues within a protein rather than between MPI and PPI molecules. In contrast to these results with pea protein, it was reported that

soy β-conglycinin (7S), dissociated soy glycinin (11S), or enzyme-hydrolyzed soy protein can interact with muscle protein to form complexes (King, 1977; Peng et al., 1982a, b; Feng & Xiong, 2002, 2003). In these studies the soy protein or its components were preheated or enzyme-hydrolyzed to induce denaturation thereby exposing reactive groups and enhancing the potential for reaction with the meat protein (King, 1977). At the high salt concentration used (0.6 M NaCl or 3.5% NaCl) in this study the denaturation of pea protein shifted to higher temperature (0.5 M NaCl, T_d = 98.7 ºC), and as a result there was insufficient denaturation of pea protein and a in lack of interactions with the meat protein. Thus, even in the presence of MTG, gel stiffness was reduced by the addition of the pea protein.

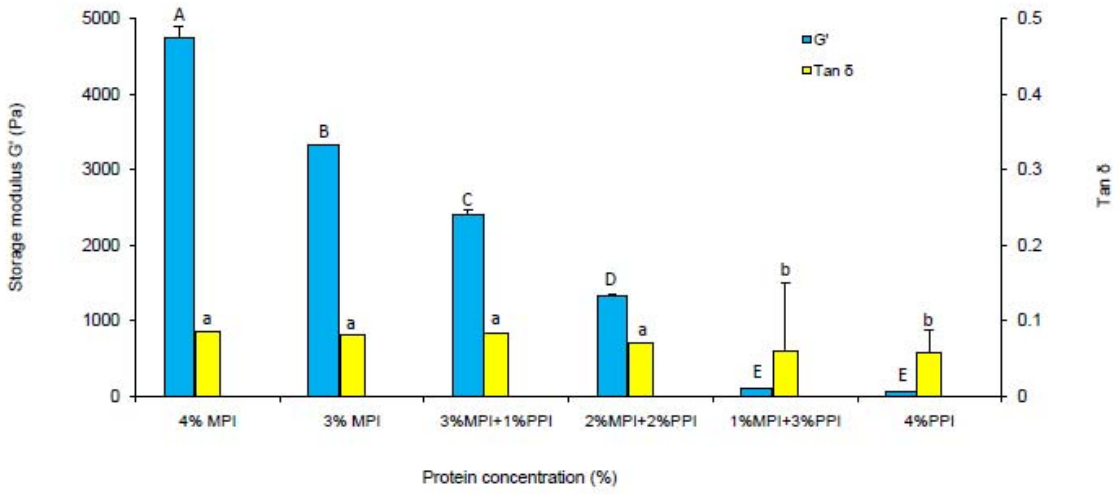

Fig. 8.17 Impact of MTG on rheological properties of various MPI/PPI protein combinations of mixed protein gels. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

The gel strength of MPI/PPI mixtures with the addition of 10 U/g protein MTG in 0.6 M NaCl was also evaluated using a penetration test and the results are shown in Fig. 8.18. Since 4% PPI did not form a gel, no penetration test could be performed. It can be seen that there were no significant differences in gel peak forces among 4%, 3%, 3% MPI + 1% PPI, and 2% MPI + 2% PPI. However, the peak force for these treatments were significantly higher than that of 1% MPI + 3% PPI. Overall these results are different from those obtained using dynamic rheology (Fig. 8.17). As noted previoiusly, Liu & Xiong (1997) indicated that the gel strength data obtained in gel penetration tests does not necessarily agree with the results of the final G' in dynamic rheological assessments, as they represent different aspects of the rheological properties of protein gels.

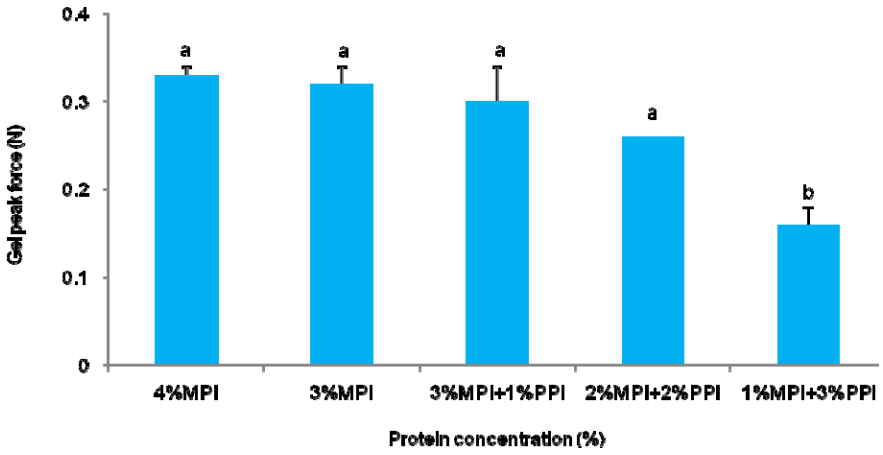

Fig. 8.18 Impact of 0.6 M NaCl on peak force of mixed protein gels. 10 U/g protein MTG was added to protein mixture (in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6) and incubated at 40 ºC for 30 min, then heated at 90 ºC for 10 min, followed by cooling down to room temperature. Note that the gel peak force of 4% PPI with 10 U MTG was too weak to be determined by texture analyzer.

Gel strength of the MPI/PPI gels with the addition of 10 U MTG/g protein at a lower salt level (0.3 M NaCl) were also evaluated using a penetration test and the results were shown in Fig. 8.19. Again the 4% PPI did not form a gel, and no data were available. It was observed that in general the peak force values for all treatments were smaller than the same treatments with 10 U MTG in 0.6 M NaCl, which indicated that higher salt concentration (0.6 M NaCl vs 0.3 M NaCl) promotes stronger protein gels.

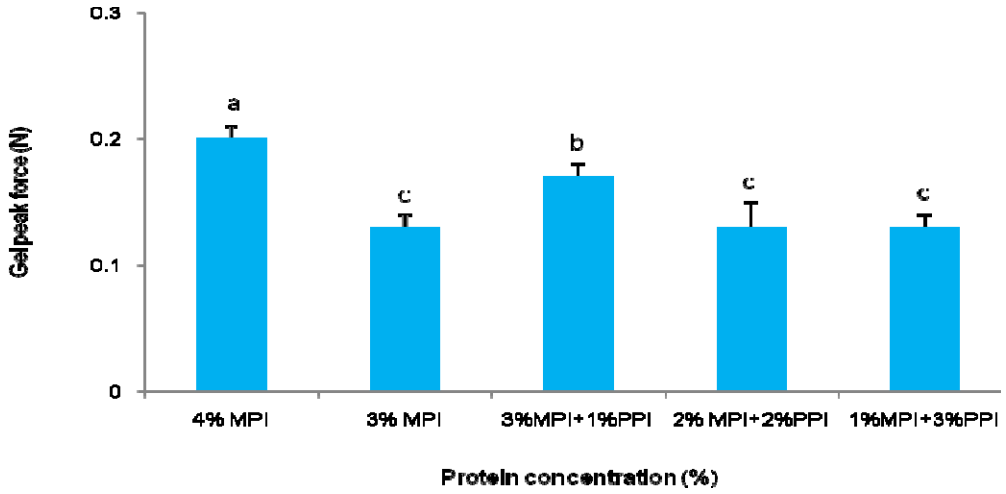

Fig. 8.19 Impact of 0.3 M NaCl on peak force of mixed protein gels. 10 U/g protein MTG was added to protein mixture (in 0.3 M NaCl, 50 mM Na_2HPO_4 at pH 6) and incubated at 40 ºC for 30 min, then heated at 90 ºC for 10 min, followed by cooling down to room temperature.

The difference is, in 0.3 M NaCl, gel peak force for 3% MPI + 1% PPI was significantly greater than for 3% MPI, although it was still less than for 4% MPI. This indicated there was interaction between MPI and PPI molecules when the T_d value of pea protein (94.5 ºC in 0.3M NaCl) was low enough to allow for protein denaturation under

the conditions used (95 ºC). With increasing proportion of PPI to MPI (2% MPI + 2% PPI and 1% MPI + 3% PPI), there was no effect on the gel peak forces in comparison with 3% MPI. This can be explained by limited interaction between MPI and PPI in combination with PPI interfering with interactions between MPI molecules.

According to Larré et al. (1992), regardless of its high content of Glu and Lys residues, pea legumin proteins in their native forms are poor substrates for transglutaminase aided crosslinking due to the close-packed globular structure. Also, this close-packed globular structure prevents interactions among pea/soy proteins and meat proteins. Therefore, in their work preheating or enzyme-hydrolysis was employed to enhance the interactions and improve gel strength. However, their work was conducted at final heating temperature of 65-73 ºC which is sufficient for meat processing, but as previously noted, is not sufficient to denature the main components of soy or pea protein. It has been reported that 7S and 11S soy proteins denatured around 75 ºC and 90 ºC (Petruccelli & Anon, 1994; Scilingo & Anon, 1996), while pea vicilin and legumin denatured around 85 ºC (Shand et al., 2007). Consequently, to open the globular protein structure through preheating or enzyme-hydrolysis prior to heat gelation is a prerequisite for better interactions between plant protein and meat protein.

Schäfer et al. (2007) has reported that by prior incubation with MTG, it is possible to modify rheological properties of heat-induced pea protein gels. Using ~ 0.2% MTG, Schäfer et al. (2007) determined a similar content of ε-(c-glutamyl) lysine isopeptide formed in commercial PPI and SPI gels (225 and 192 μmol/100 g of protein,

respectively). However, the gel strengths for these two proteins were found to be different, which indicated that crosslink location is also of importance.

To better demonstrate the impact of MTG, data for MPI, MPI+PPI, both with and without MTG have been combined and analyzed (Fig. 8.20). A soy protein isolate (SPI) was included for comparison.

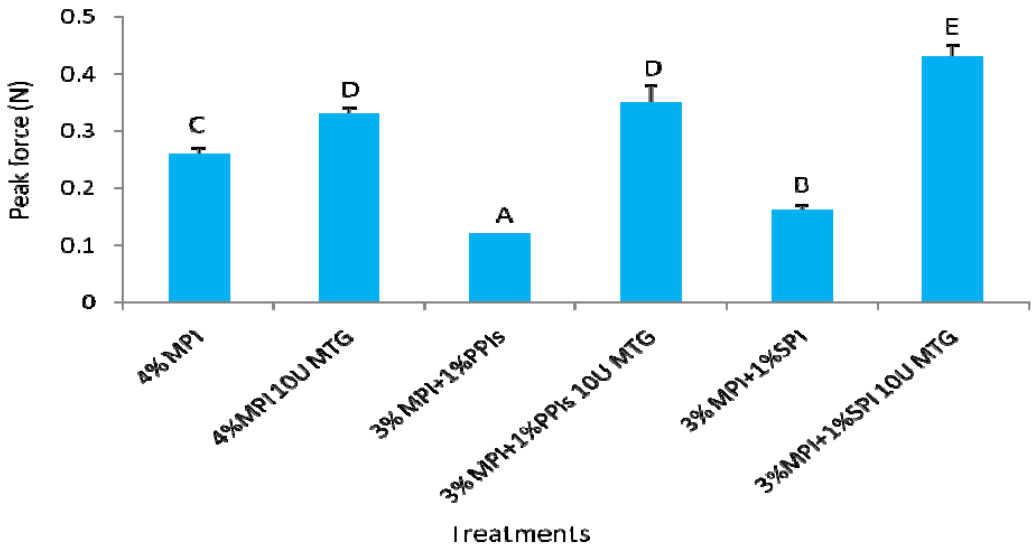

Fig. 8.20 Comparison of gel peak force of different treatments with and without MTG. Gels formed in 0.6 M NaCl, 50 mM Na_2HPO_4 at pH 6.

The gel peak force value for 3% MPI + 1% PPI with 10 U MTG (0.35 N) increased 66% and the 3% MPI + 1% SPI, 10 U MTG increased gel strength by 62.8% compared to that without MTG treatment (0.12 N). In comparison, the gel strength of 4% MPI with 10 U MTG (0.33 N) only increased 21.2% compared to that without MTG sample (0.26 N). It can be seen that the MTG treatments were more effective for MPI/PPI or MPI/SPI mixtures than MPI alone. Wang & Damodaran (1990) concluded that the

strength or rigidity of protein gels was related to the amount of intermolecular crosslinks formed in the gel network. Consequently, the results of this study suggest that more crosslinks formed in MPI/PPI or MPI/SPI mixtures with MTG compared with the pure MPI system.

Unlike the situation when MPI and PPI were mixed without MTG and the PPI acted as a nongelling filler, the interaction between PPI and MPI in the presence of MTG produced a more compatible system and a network that included both polymers was found as has been described by Ziegler & Foegeding (1990).

8.4.3.4. Effect of adding MTG on the electrophoretic and thermal properties of MPI/PPI mixtures

Electrophoretic patterns of MPI/PPI mixtures with 10 U MTG heated to 95 ºC for 10 min in 0.1, 0.3, and 0.6 M NaCl buffer were essentially the same (Fig. 8. 21 a, b, c). Only the actin band was observable for 4% MPI, and as the ratio of MPI to PPI decreased, the actin band intensity gradually decreased. In contrast, as the ratio of PPI to MPI increased, band intensities for V, L_{α}, and L_{β} gradually increased, as expected. Again no new band was found in the MPI/PPI mixtures. This disagrees with the observation for a MPI/SPI combination where two new bands formed at ~ 31 kD (Ramírez-Suárez & Xiong, 2003a). Consequently, the interaction between MPI and SPI was supported by direct electrophoretic evidence whereas no such evidence appeared for MPI and PPI combination. It should be noted that with MTG treatment, MHC and tropomyosin bands disappeared compared to samples without MTG. Also, band intensities with enzyme treatment appeared to be lighter than those without enzyme treatment.

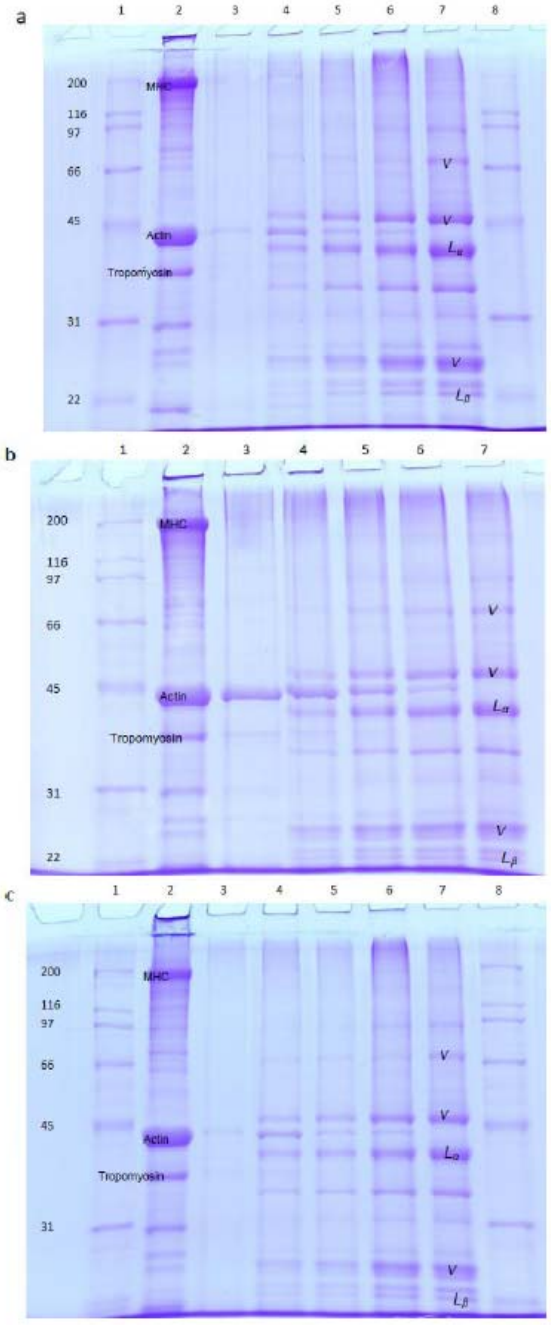

Fig. 8.21. Electrophoretic patterns of myofibrillar/pea protein isolate mixture with 10 U MTG in 0.1 M (a), 0.3 M (b), and 0.6 M (c) NaCl buffer heated to 95 °C for 10 min to form gels. Lane 1: MW standard marker, Lane 2: 1% MPI (original, not heated to 95 °C to form gel), Lane 3: 4% MPI, Lane 4: 3% MPI + 1% PPI, Lane 5: 2% MPI + 2% PPI, Lane 6: 1% MPI + 3% PPI, Lane 7: 4% PPI, Lane 8: MW standard marker. *V* represents pea vicilin proteins, L_α represents pea legumin acidic subunit, and L_β represents pea legumin basic subunit.

This difference is probably caused by large agggregates created by enzyme-catalyzed inter- and intra-molecular crosslinking. These aggregates were insoluble in the SDS-PAGE buffer and therefore did not enter separating gel. Ramírez-Suárez & Xiong (2003a) indicated that MPI/SPI crosslinking catalyzed by MTG was independent of the preponderance of one group of protein over the other. This also appears to be the case for MPI/PPI combinations because for the different MPI/PPI ratio, no bands disappeared and no new ones appeared. The only differences seen were band intensities which depended on the relative content of the protein components in the mixture.

The effect of MPI/PPI ratio with 10 U MTG incubated at 40 ºC for 30 min in 0.6 M NaCl on thermal data is shown in Table 8.2. Again the samples displayed similar thermal results as without MTG treatment, in that 4% PPI with MTG treatment had two endothermic transitions (~ 94 ºC and ~ 107 ºC) corresponding to legumin and vicilin, and 4% MPI with MTG treatment had only one endothermic transition corresponding to actin. With increasing PPI ratio, the enthalpies of two major PPI protein gradually increased, while the enthalpy of actin disappeared.

Table 8.2 DSC data of various combinations of MPI/PPI in 0.6 M NaCl buffer, after incubation with 10 U/g protein MTG at 40 ºC for 30 min

Combinations	T_{d1} (ºC)	T_{d2} (ºC)	ΔH_1 (J/g)	ΔH_2 (J/g)
4% MPI	73.3		0.44	
3% MPI/1% PPI	94.6	106.5	0.46	1.24
2% MPI/2% PPI	94.4	107.5	0.75	1.10
1% MPI/3% PPI	94.5	109.1	1.17	2.37
4% PPI	94.5	107.8	1.62	3.78

8.5. Conclusion

MPI is mainly responsible for functional properties such as gel forming ability of meat. Therefore, it is necessary to study gelation properties of MPI to see how it responds to the addition of plant protiens. As animal protein substitutes, plant proteins are usually added to comminuted meat products as minor ingredients. Thus gelation properties of meat products are dominated by MPI while plant protein PPI plays only a minor role. Since PPI denatures at a higher temperature than MPI, the effect of final heating temperatures on MPI gel stiffness was investigated at as high as 95 ºC. Final heating temperature has a significant impact on MPI gel stiffness and the maximum gel stiffness was obtained at 95 ºC. NaCl concentration and pH value also significantly influenced MPI gel stiffness. The maximum gel stiffness was obtained at pH 6, 0.9 M NaCl, but good gels also formed with salt concentrations of 0.6 and 1.2 M. Due to health concern, a lower salt concentration of 0.6 M was adopted for further study. A positive correlation was observed between gel stiffness or gel peak force and MPI concentration in the MPI concentration range of 0.5 ~ 4.5% or 2 ~ 5%, respectively. Addition of PPI to MPI resulted in a reduction in gel strength; with 0.6 M NaCl, the denaturation temperature of the PPI was higher than the temperature used for gel formation, limiting interaction between the two proteins. This study showed that with addition of appropriate amounts of MTG, the gel strength of myofibrillar proteins alone or myofibrillar/pea proteins mixture (3:1) was greatly increased. High MTG concentrations (> 15U/g protein), however, resulted in weaker gels. Although no evidence of compounds produced by the interactions between muscle and pea proteins were seen with SDS-PAGE, the great improvement in gel stiffness and gel peak force of MPI/PPI mixture (3:1) with inclusion

of MTG indicated that some ε (γ-glutamyl) lysine (G-L) crosslinking occurred between muscle and pea proteins.

In this study, it is most likely that in a mixture of PPI and MPI, the pea protein existed as dispersed liquid which acted as a non-gelling component interspersed throughout the primary MPI gel network. The addition of MTG resulted in partial crosslinking between PPI and MPI through glutamyl-lysine interactions. Consequently, MTG is useful in improving gelation properties of heat-induced MPI/PPI gels and provides new opportunities to expand the utilization of pea protein in muscle foods.

Chapter 9: General conclusion and future research considerations

9.1. Conclusion

In summary, to investigate the possibility of PPI as a functional additive in comminuted meat products, gelation properties of salt-extracted PPI and chicken MPI were investigated independently. Experiments were then conducted on the gelation characteristics of MPI/PPIs mixtures and MTG was employed to improve the gel strength and stiffness of these mixtures.

PPIs was observed to form a weak gel when compared with SPIc though it showed better gel forming ability than PPIc. Both pH and ionic had a great impact on the gel forming ability of PPIs and the maximum gel stiffness occurred at pH 4 in 0.3 M NaCl. A power law relationship was seen between protein concentration and parameters used as measures of gel stiffness. An increase in the level of proteins, and therefore the sites available for interaction between proteins, resulted in an exponential increase in the interactions formed. Slow heating and cooling rates resulted in stronger gels and cooling rate was more critical than heating rate in terms of gel stiffness. At a concentration of 0.3 M, sodium thiocyanate (NaSCN) and sodium chloride (NaCl) showed chaotropic characteristic and enhanced gel stiffness, whereas sodium sulfate (Na_2SO_4) and sodium acetate (CH_3COONa) stabilized the protein structure. The significant decrease in the G' values of pea protein gels with the addition of 3 M GuHCl and 5 M urea indicated that hydrophobic interactions and hydrogen bonds are involved in pea protein gel formation. The increase in G' with increasing PG concentration (5-20%), demonstrated hydrogen bonds and electrostatic interaction involvement. No significant influence was observed

on G' with addition of different concentrations of 2-ME, DTT, and up to 25 mM NEM, which indicated that disulfide bonds do not contribute much to pea protein gel stiffness. There was, however some indication that disulfide bonds could play a role if heating rates were low enough. The above results are in agreement with the observations of O'Kane et al. (2004c), who also observed hydrogen bonds and hydrophobic interaction, and disulfide bond under slower cooling rate (0.2 ºC/min) involved in pea and soy legumin gel formation. MTG showed great promise as a functional agent in improving the gel stiffness of heat-induced PPIs through enzyme catalyzed crosslinking and this is consistent with the result of Shand et al. (2008) who also observed a positive linear relationship between level of MTG used (0-0.7%, w/w) and shear stress and shear strain of heat-set PPIc.

The gelation properties of chicken MPI were also investigated to determine the appropriate conditions for incorporating pea protein isolate and provide a baseline to see the effects of adding pea protein with and without MTG. The final heating temperature greatly influenced MPI gel stiffness and the maximum gel stiffness was observed at 95 ºC. For muscle proteins, lower heating temperatures are normally used as these proteins denature at lower temperatures (Ziegler & Aton, 1984). The formation of a strong gel at this higher temperature allowed the incorporation of the pea protein isolate at a temperature where the pea proteins will also be denatured. Maximum gel stiffness was observed at pH 6 in 0.9 M NaCl but good gels were also formed in 0.6 and 1.2 M NaCl. A pH of 6 and 0.6 M NaCl are widely recognized as the optimal values for heat-induced gelation of chicken breast myofibrils (Xiong & Brekke, 1991; Lesiów & Xiong, 2003). The optimal pH value for gelation of chicken breast MPI in the current study was

consistent with the above observation; however, the optimal salt level shifted to 0.9 M NaCl. This was probably caused by the differences between MPI extraction procedures from different researchers. To address health concerns and keep salt addition to a mininum, 0.6 M NaCl was used for the mixed systems. As was the case with the pea protein, power law relationship described the relationships between MPI concentration and gel stiffness or gel peak force, although the concentrations used for MPI were in the ranges of 0.5 ~ 4.5% and 2 ~ 5% for gel stiffness and gel peak force, respectively. The addition of MTG improved gel stiffness but only to a point (at 12 ~ 15 U MTG /g protein). Beyond this concentration, MTG had a detrimental effect on gel stiffness or gel peak force. This is, in general, consistent with the findings in literature for whey (Truong et al., 2004), β-casein (De Jong & Koppelman, 2002), and soy protein (Nonaka, et al., 1994); however, no reports were found showing that excessive MTG resulted in reduced gel strength for meat proteins.

Using conditions that allowed for the gelation of MPI, gelation properties of MPI/PPI mixtures were studied. However, it was observed that PPIs has a detrimental effect on gelation of MPI despite the fact that temperature that promoted PPIs denaturation was used. No interaction between MPI and PPI was found through SDS-PAGE, rheology, and texture analysis. The concentration for PPIs was below that required for gel formation on its own, and when it did not interact with MPI, it simply acted as a filler and interfered with MPI network formation. For pure MPI, differential scanning calorimetry showed a transition for actin at 74.5 ºC, though a myosin transition has not been observed. For muscle proteins, the typical transition temperatures vary from 43 to 67°C for myosin and its subunits, and 71 to 83°C for actin (Amako & Xiong, 2001);

the exact temperatures are subject to pH and salt conditions (Wright et al. 1977; Xiong et al., 1987; Smyth et al., 1996). Shiga et al. (1988) observed two endothermic peaks (T_1=55.5°C for myosin and T_2=76.8°C for actin) in ground chicken breast muscle, and noted a shift of the peaks to lower temperatures upon the addition of NaCl. The transition for actin at 74.5 ºC observed in this study is in general agreement with the above literature. Although the reason why no obvious myosin transition was observed in this study is not clear, we speculate that the source of chicken breast and extraction method may contribute to this behaviour.

For the MPI/PPI mixtures, no transitions were observed for myosin and actin. In contrast, the pea proteins had denaturation temperatures of ~94 ºC and ~108 ºC for vicilin and legumin, respectively. With increasing PPI ratios, the two denaturation temperatures remained constant, whereas the enthalpies of the two transitions increased. It is possible that there was interaction among the MPI proteins prior to denaturation of the pea vicilin protein. It has been shown that the addition of sodium chloride (NaCl) or lower pH values reduce the stability of meat proteins and lowered the temperatures of denaturation (Shiga et al., 1988; Ensor et al., 1991; DeFreitas et al., 1997). Therefore, it is possible that with inclusion of 0.6 M NaCl the MPI molecules were able to interact prior to denaturation of pea proteins, thus resulting in a lack of thermal transitions for myosin and actin.

MTG was included in the MPI/PPI mixutes and the gelation properties were evaluated. With the inclusion of MTG, th salt-extracted PPI showed some ε (γ-glutamyl) lysine (G-L) crosslinking with chicken MPI as evidenced by increased gel strength, even though there was no evidence of new subunits appearing on SDS-PAGE. Several reports

indicated that interactions occurred between myosin and soy 7S β-conglycinin (King, 1977; Peng & Nielsen, 1986), partially dissociated soy 11S acidic-basic intermediate subunits and dissociated basic subunits and myosin heavy chain (Peng et al., 1982a, b; McCord et al., 1998; Feng & Xiong, 2002). Also, no new subunit bands were observed in these works. However, new bands were observed for MPI/SPI mixture in the presence of MTG (Ramírez-Suárez & Xiong, 2003a) with increased gel strength. In comparison to a gel stiffness prepared from SPIc/MPI protein mixture with MTG under the same condition, the PPIs/MPI gel was leass, but it was stiffer than the gel made from the SPIc/MPI protein mixture without MTG treatment. This is reasonable because it has been reported that pea protein can only form weak gel in comparison to soy protein (Shand et al. (2007) and with inclusion of MTG, the gel strength of PPIc was similar to SPIc and meat bologna (Shand et al., 2008). Therefore, by using MTG, PPIs represents a potential protein substitute for soy protein in comminuted meat products.

9.2. Future research considerations

Although MTG was confirmed to improve the gel stiffness of PPIs, PPIc, SPIc, and MPI, evidence of crosslinking was based on disappearance of subunits rather than identifiable new products. Determination of the ε (γ-glutamyl) lysine (G-L) content using reverse phase high performance liquid chromatography (HPLC) would provide direct evidence of crosslinking and could confirm the role of MTG as a crosslinking agent.

Also, the following research can be conducted in the future:

More meat sources (beef, pork, lamb) can be used to investigate the possibility of combining PPIs as a functional and nutritional additive in comminuted meat products.

Impact of other ingredients (eg. fat, starch) on gelation properties of MPI/PPI system could be further investigated and microscopy analysis would be useful to identify the types of networks formed in mixtures of the two proteins. Ultimately, incorporation of PPIs into fine ground chicken breast with and without MTG and the production of comminuted meat products requires consumer acceptance. Therefore, real comminuted meat products need to be developed and sensory evaluation conducted to determine the customers' acceptance.

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