EVALUATION OF PEA PROTEIN AND MODIFIED PEA PROTEIN AS EGG REPLACERS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Native yellow pea (*Pisum sativum*) protein isolates (PPIs) showed good foaming and emulsifying properties but a poor gelling characteristic. However, this can be corrected by Transglutaminase (TGase) treatment. PPIs were obtained using alkaline extraction method in which extracting pH, precipitating pH, flour–to–water ratio, and extraction time were optimized to obtain maximum yields and least change in protein functionalities. Extraction pH of 10.0, precipitating pH of 4.3, flour–to–water ratio of 1:6, and 30 minute extraction time were found to be optimum values for pea protein extraction. SDS–PAGE gels showed that the PPI had a very similar protein molecular weight profile as its original flour. TGase treatment was applied on PPIs at different pH levels from 4.3 to 7.0. The SDS–PAGE and RVA tests showed that treatment at pH 6.0 provided the best overall functionality. Large molecular weight (MW) proteins (~ 90,000 Da) and medium MW proteins (~50,000 – 80,000 Da) were the main substrates for TGase catalyzed reaction whereas most low MW the proteins (< 45,000 Da) were not involved. RVA results indicated that treatments at pH 6.0 and 7.0 had the highest viscosities but the treatment at pH 6.0 had better stability and consistency. Functionality tests indicated that modified PPIs possessed a better viscosity profile than the native PPIs but no improvement in gelling capacity and only minor impact on foaming and emulsifying properties. PPIs performance greatly depended on their final pHs. The foaming capacity, foaming stability, and emulsion capacity were significantly improved when the final pH of PPIs was adjusted from 4.3 to 7.0. The overall sensory evaluation results suggested that TGase–treated PPIs and PPIs were not yet able to replace egg in the cake system. Only PPI can replace egg in the cookie system. TGase–treated samples had

a lower acceptability due to an “off–taste” and a “strange” flavor. Future work, therefore, should study TGase combined with other treatments to further improve PPIs functionalities. Purification should be integrated into extraction process and other food systems should also be included to extent the scope and role of modified PPIs in food industry.

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GENERAL INTRODUCTION

Peas are small seeds of the legume *Pisum sativum*, which are rich in protein, starch, fiber, vitamins and minerals. Pea plants are grown in many regions of the world where the climate is temperate. On worldwide scale, pea is grown on over 25 million acres (Schatz and Endres 2009). Approximately, 756,000 acres of field peas were grown in the United States in 2010 (USDA Ag Statistics http://www.nass.usda.gov/). Russia, China, followed by Canada, Europe, Australia and the United States are major producing countries of field peas. However, Europe, Australia, Canada and the United States are major exporters (Schatz and Endres 2009). Although originated in southwestern Asia, possible northwestern India, Pakistan or adjacent areas of former USSR and Afghanistan (Kay 1979), yellow field peas (*Pisum sativum* L.) are now a major pulse crop in western Canada. Canada is one of the world's leading field pea exporters (Wang et al. 2003). Traditionally, peas have been classified into two common forms, field peas or dry peas and fresh or succulent peas (Schatz and Endres 2009). Succulent peas are peas consumed in forms of fresh, frozen or canned while dry peas are normally referred to dried split peas or dried whole peas.

The most common applications of peas as human foods are ingredients in soups, snacks, pudding, stew or sprouts. Not only being used as food for human, peas are also usually used in feeding animal as leguminous constituent in the mixture with cereal or canola meal to enhance the protein portion of animal diets (Government of Manitoba 2010). However, peas have other potential functional properties that could be exploited and brought into use in applications that benefit not only consumers but also producers to promote the sustainable development and expansion of the pea growing areas. Recently,

pea starch has been extracted and used as an ingredient in making bio–plastics which are environment friendly and have a lot of promising applications in the near future (Chen et al. 2009).

Unlike peas, egg are recognized as gelling, emulsifying, foaming and thickening agents and have already been well studied and characterized by many scientists. Currently, egg has been widely used in food industry and played an irreplaceable role as a functional ingredient in many food products. However, recently, public concerns have been rising significantly about egg allergen and negative effect of consuming too much egg. Egg allergy is one of the most common food allergies, affecting about 1% to 2% of young children (Eggesbo et al. 2001; Sicherer and Sampson 2006). Also, since one egg yolk contains about 200 mg of cholesterol, the American Heart Association has an official suggestion in which people with heart disease should consume no more than 2 egg yolks per week (American Heart Association http://www.heart.org/). Besides, fluctuation in cost of egg (USDA AMS Poultry Market News and Analysis www.ams.**usda**.gov/mnreports/pywgraph1.pdf) would reduce the interest of manufacturers in using egg in their products. Fully or partially replacing egg ingredient in food formulas using pea protein isolate will therefore be of interest to the food industry.

Currently, some commercial pea protein isolate products have already been introduced to consumers. They are usually spray–dried but none of these products are from a freeze drying process. Apparently, certain high temperature treatment will denature the protein and in some particular situations, denaturation of proteins is necessary for their functionalities. However, freeze drying method which is believed to best preserve the native functionalities of pea protein (Shand et al. 2007) was used in this

project in order to maintain the native pea protein structure to investigate the effect of transglutaminase (TGase) on pea protein isolate. Protein isolates were prepared in the laboratory from field yellow split pea flour provided by Northern Crops Institution (NCI) using method adopted from Sumner et al. (1981) with little modification. The best isolates were tested for "intrinsic" functionalities and compared to those of commercial pea protein isolates available on market. In this project, Nutralys F85M (Roquette) pea protein isolate was used as the benchmark product in functionality comparison. Native functionality of pea protein was studied using methods from different sources which included methods used currently in our lab for foaming and emulsion, and methods proposed by Bildstein et al. (2008), for gelling, and based upon the result, further modification using enzyme treatment was conducted.

In order to be a good egg replacer, a pea protein isolate should possess not only a good foaming capacity and stability but also a good gelling ability. Two types of enzymes were considered in this study. The TGase is the transferase to look at due to its ability to link different individual protein molecules to form a big complex protein which helps increase the gelling capacity of pea protein isolate (Sun and Arntfield 2011a, b). Theoretically, TGase catalyzes the formation of a covalent bond between a free amine group (e.g., protein/peptide–bound lysine) and the gamma–carboxyamide group of protein/peptide–bound glutamine (Ohtsuka et al. 2000). Currently, there are two commercial TGase products available on the global market and the one that was made by Ajinomoto, Japan was chosen.

Another enzyme system used in this research was a mixture of fungal amyloglucosidase and fungal α–Amylase that are expected to break down soluble starch

in the protein extracts. The use of such enzymes was based on the increased protein concentration in the protein isolate (Bildstein et al. 2008) and to eliminate the effect of starch remnant in pea protein isolate on the protein functionality.

This project focused on the protein portion of peas and evaluation of such a portion as an egg replacer in commercial food products including bakery items. Cakes and biscuits, i.e. cookies, were the two food systems used to test the functionality of modified pea protein isolate as an egg replacer. Results from these experiments demonstrated the functionality of the pea protein in real food systems and how they can be compared to egg protein in terms of foaming capacity and emulsifying capacity. Based on literature, we expected that the pea protein will function similarly to eggs in cake and cookie systems.

The success of the project will positively impact many parties. Using new ingredient as an egg replacer in food systems that might be more cost effective, have a better health image, and provide a good choice for consumers who cannot eat products containing egg, i.e. people with egg allergy or coronary heart diseases, or vegans, is of interest to food manufacturers. Pea growers will benefit as new markets for pea ingredients will be developed.

HYPOTHESIS

Untreated pea protein isolate was reported to have good foaming capability and foaming stability but are poor in gelling ability, suggesting pea proteins are not similar to egg proteins. However, if an appropriate method is used to extract protein from pea (*Pisum sativum*) and suitable enzymes are used to modify pea protein extract, their functionalities can be enhanced and therefore can be used to replace egg in some food systems.

OBJECTIVES

The objectives of this research were to:

1) Optimize the extraction protocol based on alkaline extraction/isoelectric precipitation method for yellow pea to maximize yield while retaining protein nature,

2) Modify PPIs to improve gelling functionality by applying TGase treatment,

3) Characterize PPIs and TGase–treated PPIs extracted from optimized process, and

4) Evaluate PPIs and modified PPIs as egg replacers using physical and sensory methods.

LITERATURE REVIEW

1. PEA

1.1. General information

Peas are small seeds of the legume *Pisum sativum* that are rich in protein, starch, fiber, vitamins and minerals. Field pea is a cool–season legume crop that is grown on over 25 million acres worldwide (Schatz and Endres 2009). In the United States, approximately 756,000 acres of field peas were planted in 2010 (USDA Ag Statistics http://www.nass.usda.gov/). Before 1997, Washington and Idaho were the major producers (i.e. highest acreage) of dry peas across the United States but from 2002 to 2010, North Dakota ranked number one in pea growing acreage and pea production in the United States (USDA Ag Statistics http://www.nass.usda.gov/). Approximately 60% of total pea growing acres in the United States were in North Dakota by 2007, which represents a 301.9% increase in pea growing acreage in North Dakota from 2002 to 2007 (USDA Ag Statistics http://www.nass.usda.gov/). There has been only a slight change in acreage recently and the trend is very stable as shown in Figure 1. The highest gains in acreage and production were observed with yellow peas. In 2008, about 528,089 acres of yellow peas were grown resulting in a production of 307,826 MT, approximately 50% higher than the second largest contributor, i. e. green peas, which had a production of 208,784 MT (USA Dry Pea and Lentil Council http://www.pea–lentil.com/).

The green– and yellow– cotyledon varieties are commonly grown in North Dakota and they are used for human consumption as dry split field peas. In North Dakota, pea yields are similar to hard red spring wheat yields (N.D. Agricultural Experiment Station http://www.ag.ndsu.nodak.edu). Pea yields in North Dakota vary from as low as

1,600 lbs/acre to 2,400 lbs/acre depending on weather conditions (Figure 1). A cool growing season is necessary for optimum yields whereas hot weather during flowering may reduce seeds per ovary (N.D. Agricultural Experiment Station http://www.ag.ndsu.nodak.edu).

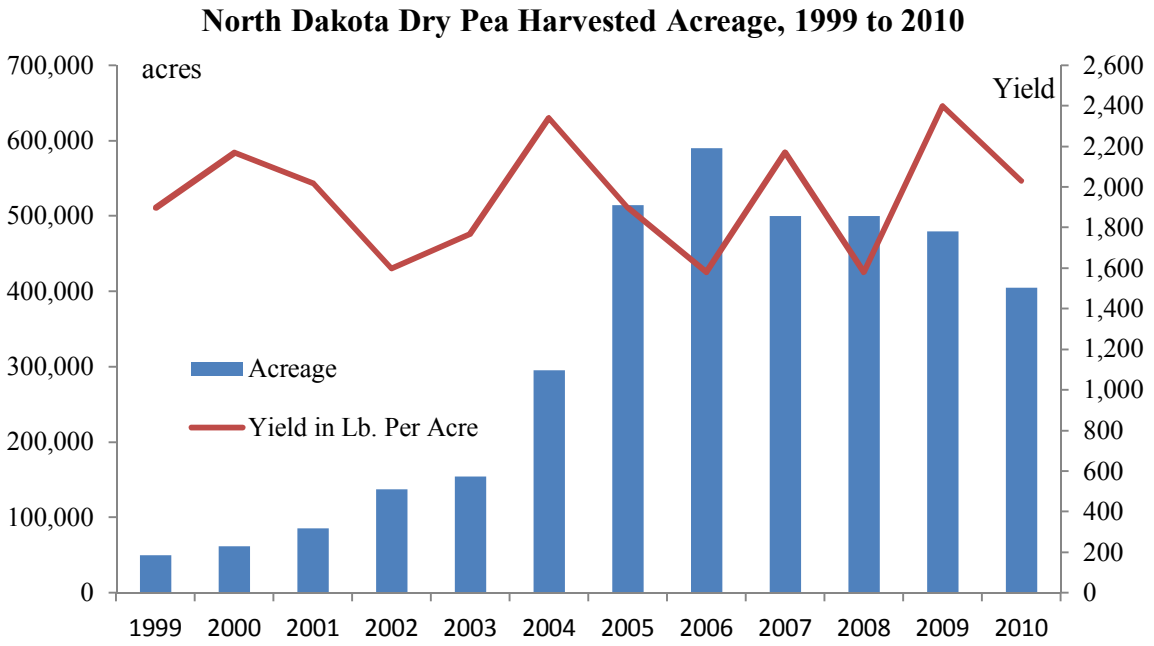


Figure 1. North Dakota dry pea growing acreage and yield from 1999 to 2010*.
* Compiled from the USDA Ag Statistics http://usda.mannlib.cornell.edu.

Most (73.5%) of US dry pea production was for exporting purposes while relatively small amount of dry pea is used for domestic consumption (USDA, http://www.ers.usda.gov/briefing/dyrbeans). India is the leading import market for US pea with 22% of the total volume shipped, following by Kenya (18%), Spain (7%), Pakistan (6%) and Canada (5%), during the first 2 months of the 2011/12 marketing year, i.e. July – August (USDA, http://www.ers.usda.gov/briefing/drybeans/).

1.2. Pea protein

Peas are an important source of food proteins (Boye et al. 2010). Field peas contain approximately 21–25% protein and have high levels of the essential amino acids, lysine and tryptophan, which are relatively low in cereal grains (Schatz and Endres 2009; Boye et al. 2010). Protein contents up to 34.7% were found in some specific varieties such as Lencolen (El–Adawy et al. 2003; Boye et al. 2010). The major proteins found in pulses are globulins and albumins. In pea, albumin and globulin represent 15–25% and 50–60% of the total protein, respectively (Gueguen and Barot 1988). Others have reported pea seed albumins to account for roughly 15–40% of the total proteins in the cotyledon (Rao et al. 1989; Swanson 1990) and remaining proteins being largely globulins (Boye et al. 2010).

Albumins are water soluble while globulins are salt soluble. Two major albumin proteins have been identified in peas. The major albumin protein contains two polypeptides with molecular weights of ~25,000 Da whereas the minor albumin protein has a molecular weight of approximately 6,000 Da (Rao et al. 1989). The major globulins found in pulses are legumins (11S) and vicilins (7S). 11S Legumins have hexameric quaternary structures with acidic (molecular weight of ~40,000 Da) and basic (molecular weight of ~20,000 Da) subunits (Boye et al. 2010). The 7S vicilins have a trimeric structure with molecular weights of 175,000–180,000 Da (Boye et al. 2010). Globulin proteins have important roles in many foodstuffs, both because of their nutritional value and of their contribution to food texture (Van Kleef 1986). One of the most important functional properties of the globular proteins is gelation (Ikeda and Nishinari 2001).

Convicilins, prolamins and glutelins are other minor storage proteins found in pulses (Croy et al. 1980; Gupta and Dhillon 1993; Saharan and Khetarpaul 1994).

In terms of amino acid composition, pea protein has an essential amino acid to total amino acid ratio of 0.46 for green peas (Iqbal et al. 2005) and 0.47 for pea protein isolate (Pownall et al. 2010), while this ratio in soybean is 0.36 (Wang and Cavins 1989). Pea albumins contain more of the essential amino acids tryptophan, lysine, threonine, cysteine and methionine compared to the pea globulins, while the globulin proteins are rich in arginine, phenylalanine, leucine and isoleucine (Swanson 1990). Methionine and cysteine are the two most limiting essential amino acids (Table 1).

Table 1. Amino acid composition of pea (*Pisum sativum*) protein.

Amino acid	Essential amino acid		Amino acid	Non–essential amino acid	
	Ref. [A]	Ref. [B]		Ref. [A]	Ref. [B]
Isoleucine	3.33	3.89	Alanine	4.27	4.83
Leucine	6.58	7.84	Aspartic acid	10.68	11.16[b]
Lysine	6.84	6.25	Cysteine	1.55	0.35
Methionine	1.03	1.60[d]	Glutamic acid	16.92	18.46[c]
Phenylalanine	4.19	5.17	Glycine	4.32	4.82
Threonine	3.59	4.46	Proline	3.76	4.64
Tryptophan	0.94	0.61	Serine	4.79	5.71
Valine	3.89	5.11	Tyrosine	3.16	3.34
Arginine[a]	6.84	7.93			
Histidine[a]	2.52	2.33			

[A] Unit: g amino per 16 g N (Leterme et al. 1990), [B] Unit: g/100 g protein (Khattab et al. 2009). [a] Conditionally essential, [b] Aspartic acid + asparagine, [c] Glutamic acid + glutamine, [d] Methionine + cysteine.

Pea protein has a very similar amino acid profile to that of soy protein. Field peas also contain 5 to 20 percent less of trypsin inhibitors than soybean, which allow them to

be used directly for livestock without having to undergo a heat treatment process (Schatz and Endres 2009). However, pea protein digestibility is lower than that of soy protein, i.e. 60.4–66.5% vs. 71.8% (w/w) (Bishnoi and Khetarpaul 1994; Han et al. 2007).

The primary interest in pea has been in the animal feed industry according to Government of Manitoba (2010). Pea flour is usually used as a protein component in mixture with cereal flour (Huisman and Van Der Poel 1994). Only small amount of peas are used in the human diet, in different forms including but not limit to fresh pea, cooked whole pea, split pea, frozen pea, canned pea, roasted pea, pea sprout, or dried pea.

Although peas are well known for both of their high carbohydrate and protein contents, initial interest in pea flour seems to be from their high starch content. Extensive studies about pea starch, their functionalities, and their effects on multi systems have already been reported (Swain and Dekker 1966; Ring 1983; Ratnayake et al. 2002; Ma et al. 2008; Chen et al. 2009; Simsek et al. 2009; Tan et al. 2009). Studies about pea starch will not be presented in this dissertation because the scope of the research is on pea proteins.

Pea protein was studied early in the last century when Satow (1918) invented a way of making varnish from pea protein. However, scientists' interest in pulse protein increased significantly in the mid–20th century. Short thereafter, pulse protein in the form of either flour or protein isolate had been evaluated in many different food systems such as in infant food, bakery products and fried products (Stanton et al. 1966; Kurien et al. 1972; Besrat 1981). Again, the pulse component in such formulas was aimed to improve nutritional values and there was very limited understanding about pulse protein functionality in such food systems. Recently, a number of studies about pea protein

functionalities in model systems have been completed, revealing an interesting picture about pea protein functionality compared to common soybean protein isolates (Sumner et al. 1981; Periago et al. 1998; Rangel et al. 2003; O'Kane et al. 2005; Kaur et al. 2006; Shand et al. 2007; Shand et al. 2008; Sun and Arntfield 2011a,b).

Generally, gelling capacity of pea protein isolate was worse than that from soybean protein (Bildstein et al. 2008), which means gels obtained from peas were weaker and had less structure compared to those formed from soy protein. However, pea protein isolates proved to be a better emulsifier and foaming agent at pH 7.0 compared to soy protein isolates at the same pH levels (Bildstein et al. 2008). The former characteristic, however, can be improved by applying enzymatic treatments on pea protein isolate. Transglutaminase treatment can improve the gel strength (Shand et al. 2008; Sun and Arntfield 2011a) while acid proteases have a positive effect on emulsification capacity (Periago et al. 1998), making enzyme treated pea protein isolate a potential functional material as gelling, foaming and emulsifying agents compared to other material, e. g. egg white protein, soy protein isolates. Although, Bildstein et al. (2008) noted a reduction in foam stability, these authors did report that enzyme modified lentil flour produced acceptable pound and sponge cakes, indicating that the foaming and emulsifying properties of modified lentil flour observed in model systems were still retained in a real food system, i.e. the sponge cake. However, the limitation of Bildstein et al (2008) study was that lentil flour was an additional component in cake formula and was not intended to replace egg. Therefore, we will, in this project, evaluate the pea protein isolates and modified pea protein isolate as a replacement for egg component in cake and cookie formulas to confirm if the pea protein isolates and modified pea protein

isolates are capable of retaining foaming, emulsifying and gelling properties in real food systems as observed in model systems.

Moreover, by using pea protein isolate along with cereal flour (wheat flour), we can also improve the protein quality of baked products. The lysine content falls in the range of 6.9 –8.2% of total protein (Bressani and Elias 1988; Huisman and Van der Poel 1994), which is high enough to compensate for the deficiency of lysine in common cereal flours. This makes the whole food product an excellent source of protein and comparable to animal protein sources.

2. EGG PROTEIN

Egg has been a human food since ancient times and is considered one of nature's nearly perfect protein foods (Belitz et al. 2009). Of all eggs, chicken eggs are the most important; those of others such as duck, geese, and quail are of less significance (Belitz et al. 2009). A medium chicken egg usually weighs about 58 g of which water accounts for approximately 74%, protein (~12%) and lipids (~11%) (Belitz et al. 2009). Chicken egg white proteins have been extensively utilized as an important ingredient in food processing because of their unique functional properties such as gelling, foaming, emulsifying, heat setting and binding adhesion (Mine 1995). Protein (albumen) is a major nutritional component of egg white which constitutes ~ 9.7–10.6% (w/w). Carbohydrate only accounts for 0.5–0.6% and exists either in free form or bound form with protein. Glucose, at 0.5%, accounts for 98% of total free carbohydrates (Mine 1995). Lipid in egg white is very negligible and accounts for just 0.03% (Belitz et al. 2009).

The protein composition in egg white has been well studied and major components are summarized in Table 2. The three main protein components, i.e.

ovalbumin, ovotransferrin and ovomucoid, account for nearly 80% of the overall protein content in egg white. Ovalbumin is the main albumen protein that was crystallized by Hofmeister in 1890. It is a glycophospho–protein, which is relatively readily denatured. This is an interphase denaturation that occurs through unfolding and aggregation of protein molecules. In contrast, ovotransferrin or conalbumin is not denatured at the interphase but coagulates at low temperature and has the ability to retard growth of microorganisms. Ovomucoid has 9 disulfide bonds in its structure and is stable against heat coagulation. It can inhibit bovine but not human trypsin activities (Belitz et al 2009).

Table 2. Composition and properties of major egg white proteins*.

Protein	albumen (% db)	pI**	Molecular weight (kDa)	Characteristics
Ovalbumin	54.0	4.5	44.5	Glycophospho –protein
Ovotransferrin	12.0	6.1	77.7	Binds metallic ions
Ovomucoid	11.0	4.1	28.0	Inhibits trypsin
Ovomucin	3.5	4.5–5.0	$5.5–8.3x10^3$	Viscous
Lysozyme	3.4	10.7	14.3	lyses some bacteria
G2, G3 globulin	8.0?	5.5–5.8	49.0	
Avidin	0.05	10.0	68.3	Binds biotin

* Adopted from Mine (2005) with data compiled from Powrie and Nakai (1986).
** pI: isoelectric point.

When heated, egg white proteins denature and form a thermal–irreversible coagulum, which gives products such as meringue and angel food cake their characteristic textural qualities (Mine 1995). Egg white begins to coagulate at 62^0C and is greatly influenced by pH. Egg white gels at room temperature in solutions at or above pH 11.9, though after a while the gel will liquefy (Belitz et al. 2009). All egg white proteins will coagulate when heated except ovomucoid. During the thermal denaturation and

aggregation of egg white proteins, the stable intermolecular antiparallel β–sheet structures form (Painter and Koenig 1976; Kato and Takagi 1988; Mine et al. 1990). The formation of a stable intermolecular β sheets plays the central importance in the thermal denaturation and aggregation of egg white (Mine 1995).

Besides the excellent gelling capacity, egg white is also known as an excellent food foaming agent. Globulin constituent is the most important protein contributing to the excellent foaming properties of egg white, followed by ovalbumin, ovotransferrin, lysozyme, ovomucoid and ovomucin (Johnson and Zabik 1981). In contrast, Belitz et al. (2009) stated that ovomucin plays a more important role in egg foaming ability as it forms a film of insoluble material between the liquid lamella and air bubbles, thereby stabilizing the foam. At the natural pH of fresh egg, the basic protein lysozyme is positively charged and can interact with negatively charged proteins to form electrostatic interactions (Phillips et al. 1989), which contribute to the excellent foaming properties of egg white and most importantly to the heat stability characteristics (Mine 1995).

Although having been an important functioning agent in food industry, egg causes some health problems to particular groups of consumers. Egg allergy is one of the most common food allergies, affecting about 1% to 2% of young children (Eggesbo et al. 2001; Sicherer and Sampson 2006). Milk allergy is the number one in prevalence, followed by egg allergy. However, many studies showed that egg allergy is the most common food allergy in children with atopic dermatitis (Sampson 1983, Sampson and McCaskill 1985; Sampson and Scanlon 1989). Although most children are likely to develop egg tolerance at their late childhood, some with an egg IgE greater than 50kU/L may never out–grow their egg allergy (Savage et al. 2007).

Egg yolk contains 32.6% lipid (Belitz et al. 2009). Cholesterol, cholesterol esters and other non triacylglycerols, and phospholipids compounds account for 6% of total lipids of which sterols are about 4% of total lipids (Belitz et al. 2009). Cholesterols account for 96% the sterols thus making egg yolk the highest source of cholesterol in all foods (Belitz et al. 2009). About 186 mg of cholesterol is found in one large egg (USDA, http://www.nal.usda.gov). Due to the high level of cholesterols in egg yolk, egg consumption in older Americans decreased by 46 percent in males and 29 percent in females from 1977 to 1996 (Gerrior 1999). Even though recent clinical studies have shown that egg consumption and serum cholesterol concentrations are not directly related (Dawber et al. 1982; Green and Jucha 1986; Ginsberg et al. 1994; Kritchevsky et al. 1998), the American Heart Association still has an official suggestion that people with heart disease should consume no more than 2 egg yolks per week (American Heart Association http://www.heart.org/).

Food manufacturers have concern about the high cost of eggs. The egg price is not stable and changes very quickly in a short period of time (Figure 2). This causes the food manufacturers and other egg users in food industry problems in long term planning, cost calculation and budgeting.

In contrast, the season average price for dry field pea in 2009 was only $8.99/cwt (USDA http://usda.mannlib.cornell.edu), which is much cheaper than egg and soybean prices. Fully or partially replacing egg ingredient in food formulas using pea protein isolate will therefore be of interest to the food industry. Using new ingredient as an egg replacer in food systems that might be more cost effective, have a better health image, and provide a good choice for consumers who cannot eat products containing egg, i.e.

people with egg allergy or coronary heart diseases, or vegetarians, is of interest to food manufacturers. Pea growers will benefit as new market for pea ingredients will be developed.

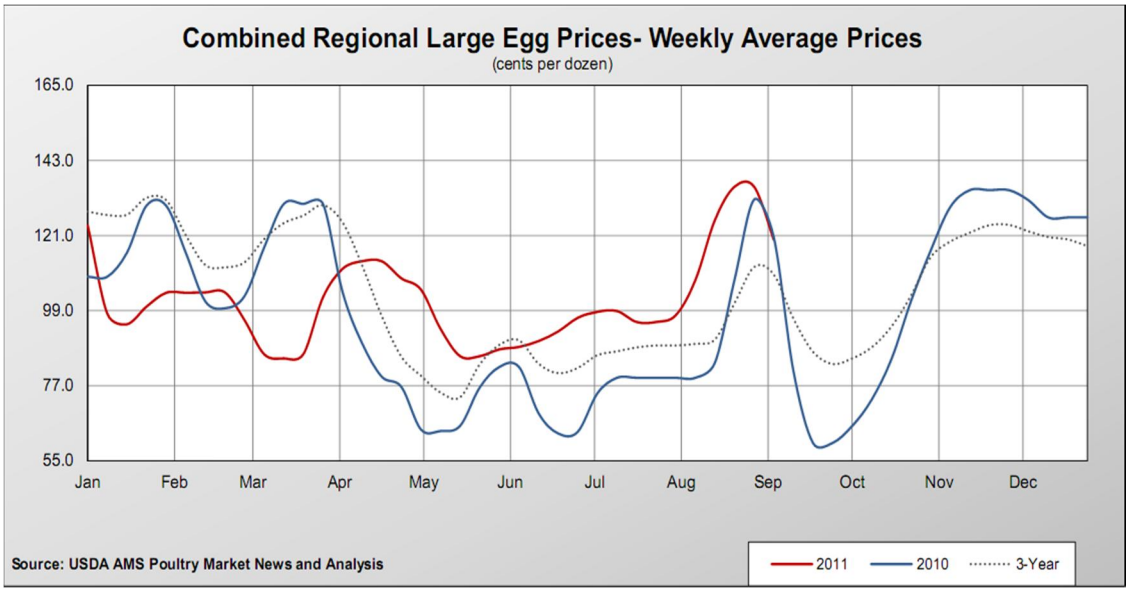


Figure 2. Egg prices in 2010 and 2011 on weekly basic.
Source: www.ams.**usda**.gov/mnreports/pywgraph1.pdf.

3. PROTEIN EXTRACTION METHODS

Six techniques have been commonly used for processing pulse protein flours, concentrates and isolates, including air classification, water extraction, salt extraction (i.e. micellization), ultrafiltration, acid extraction, and alkaline extraction/Isoelectric precipitation (Boye et al. 2010). Each of these methods has advantages and disadvantages, thus no one method is ideal.

Air classification is a milling technique that allows the fractionation of flour into high starch and high protein fractions. Milling of pulses results in flours having particles of two discrete sizes and densities (Boye et al. 2010). The light fine fraction contains high

protein content whereas the heavy coarse fraction consists of most starch. This method is simple and does not require chemicals; however, the purities of both starch and protein fractions obtained from air classification are often lower than those obtained with aqueous extraction processes (Boye et al. 2010). This method also requires several milling cycles (Gueguen et al. 1984) to obtain complete cellular disruption and to maximize protein and starch separation (Tyler and Panchuk 1982). Typically, protein fractions obtained from first classification contain 49% to 75.1% protein depending on type of pulses (Tyler et al. 1981; Elkowicz and Sosulski 1982) and moisture (Tyler and Panchuk 1982). Other researchers reported slightly lower values that range from 40% to 62% (Patel et al. 1980; Aguilera et al. 1984; Gujska and Khan 1991a, b).

Water extraction of pulse has also been studied. Normally, extraction protocols using multiple water extractions improve the recovery rate (Martin–Cabrejas et al. 1995; Cai et al. 2001). Protein content in final extract was found to be from 54% to 67% for chickpea and smooth pea, respectively. No recovery rate was reported but it would be low considering the type of extraction.

Salt extraction or micellization process in based on the salting–in and salting–out phenomenon of food proteins (Boye et al. 2010). In this process, after extracting protein at high salt concentration at desired ionic strength, solution is diluted to promote protein precipitating which can then be recovered by centrifugation or filtration. A high protein content of 87.8% was reported in chickpea protein extract by Paderes–Lopez et al. (1991). Lower range from 74.7% to 84.2% was obtained in common bean protein extract using similar methods (Marquez et al. 1996).

The ultrafiltration technique is commonly used in combination with alkaline or acid extraction. The supernatants derived from mentioned techniques are used as input for ultrafiltration. A protein content of 94.1% and 89.5% from faba bean (*Vicia faba equina* L. cv. Diana) and pea flour (*Pisum sativum* L. cv. Trapper), respectively, were reported by Vose (1980) using ultrafiltration technique. The separation efficiency of this method greatly depends on type of membrane, the molecular weight cut–off, and the volume concentration ratio and filtration conditions (Boye et al. 2010).

Acid extraction involves protein extraction under acidic conditions (Boye et al. 2010).Various protein purities has been reported. Alli et al. (1993) reported that using acid extraction method, they were able to obtain the protein isolate containing 95.7% of protein from white kidney bean. Acid extraction of pin–milled faba bean (*Vicia faba–equina* L. cv. Diana) and pea (*Pisum sativum* L. cv. Trapper) resulted in extracts with protein contents of 91.2% and 91.9% for faba bean and pea, respectively (Vose 1980). However, only 50% of protein content was reported in lima bean using the acid extraction method (Ologhobo et al. 1993). The processing conditions greatly influence the yield and purity of finished products, resulting in differences in reported protein purities (Boye et al. 2010).

The alkaline extraction/isolectric precipitation is the most common method found in literature especially for legume protein extraction (Flink and Christiansen 1973; Chakraborty et al. 1979; Bahnassey et al. 1986; Duszkiewicz–Reinhard et al. 1988; McCurdy and Knipfel 1990; Paredes–Lopez et al. 1991; Ologhobo et al. 1993; Fernandez–Quintela et al. 1997; Szymkiewicz and Jedrychowski 1998; Rubio et al. 1999; Freitas et al 2000). Similar to acid extraction technique, protein purity and yield are easily

affected by processing conditions (e.g., temperature, time, solvent–to–flour ratio, condition and protein solubility of the starting material, type of equipment and process used, g forces used for centrifugation, laboratory vs. pilot–scale extraction, batch vs. continuous extraction, etc.) (Russin et al. 2007). At good processing conditions, protein content in protein isolate can range from 80% to 94% depending on types of legumes (Flink and Christiansen 1973; Chakraborty et al. 1979; McCurdy and Knipfel 1990; Paredes–Lopez et al. 1991; Fernandez–Quintela et al. 1997). However, changes in processing conditions may result in very low protein purities, i.e. 46.4% to 59.3% (Bahnassey et al. 1986; Ologhobo et al. 1993).

Water extraction, salt extraction, ultrafiltration, acid extraction, and alkaline extraction/Isoelectric precipitation can be referred to as one category, typically known as aqueous extraction. Beside aqueous extraction and physical extraction, i.e. air classification, organic solvent extraction method has been used. Yu et al. (2010) used acetone, trichloroacetic acid (TCA) and the combination of these two to extract protein secreted from *Aspergillus fumigatus*.

The aqueous extraction of soluble components is the basis of several industrial processes including protein extraction. These methods possess some advances over other methods, e.g. organic solvent method. First, it is more environmentally friendly and safer since it does not use any type of organic solvent (Rosenthal et al. 1998). Second, it is generally less expensive compared to the organic solvent method. Finally, it is easier to handle and does not require too much skill. Although, in most cases, the aqueous extraction methods cannot extract all the protein from the raw material, they are now becoming very common extraction methods and are widely used in the industry for

various materials such as soybean (Rosenthal et al. 1998), sunflower (O'Connor 1971; Arntfield 2004) or pea (Sumner et al. 1981). Sumner et al. (1981) proposed an aqueous extraction method to extract protein from field peas based on alkaline extraction (pH 8.5) followed by acidic precipitation (pH 4.5). The technique takes advantage of the solubility of legume proteins which is high at alkaline pH and low at pH values close to the isoelectric point (~pH 4 to 5) (Boye et al. 2010). These authors concluded that chemical analysis, functional properties, color and flavor of the dried pea isolates compared favorably with the soy protein counterparts and freeze–drying and spray–drying resulted in pea isolates with the highest emulsification and water absorption values (Sumner et al. 1981). Among different drying methods, spray drying produced the best foaming, color and flavor properties.

Recently, Bildstein et al. (2008) studied the enzyme–based aqueous extraction process for vegetable proteins to be applied in bakery products and their works suggested that with the use of enzyme during the extracting step, protein extracts showed similar or even better functional properties regarding foaming and emulsifying capacities, heat stability and gelling properties compared to soy and pea protein extracts. α–Amylase and amyloglucosidase are an enzyme system used in the research to digest starch. α–Amylase cleaves starch molecules internally and thus is called an *endo*–enzyme. α–Amylase hydrolyzes α–1,4 glucan linkages randomly but not α–1,6 glucan linkages, causing a rapid decrease in viscosity of starch slurries (Campbell–Platt 2009). The starch cleavage by α–Amylase results in oligosaccharides of 6–7 glucose units (Belitz et al. 2009). The oligosaccharides are main substrates for amyloglucosidase hydrolysis. Amyloglucosidase is an *exo*–enzyme and can hydrolyze both α–1,4 and α–1,6 bonds to yield glucoses

(Campbell–Platt 2009). It starts at the non–reducing end of α–1,4 glucans and liberates β–D–glucose units (Belitz et al. 2009). Amyloglucosidase cleaves α–1,6 bonds 30 times slower than α–1,4 bonds (Belitz et al. 2009). Both α–Amylase and amyloglucosidase can be derived from difference sources including plant and microorganisms, i.e. bacterial, yeasts, and molds.

Another aqueous extraction technique used to extract pea protein was salt extraction (Sun and Arntfield 2011b). Sodium chloride 0.3 M solution was employed to extract pea protein from pea flour, followed by centrifuge, precipitation and dialysis steps. Freeze dry method was also used in proposed enzyme–based aqueous extraction method of Bildstein et al. (2008). According to Sun and Arntfield (2011b), pea protein isolated extracted by this method showed much stronger gel strength compared to commercial pea protein isolate but still lower than that of commercial soy protein isolate. Combining these findings from different authors, the idea of considering both extraction conditions and modifications as a means to improve functionality of yellow split pea system should be considered.

4. TRANSGLUTAMINASE MODIFICATION OF PROTEIN

Transglutaminase (TGase, E.C. 2.3.2.13) is a very common enzyme used in food industry especially in processed meat and in noodles to improve product texture. TGase is an enzyme often used for the cross–linking of food proteins (Sun and Arntfield 2011a). Most food proteins, such as legume globulins, wheat glutenin and gliadin, egg yolk and egg white proteins, meat actins and myosins, gelatin, collagen, milk caseins, α–lactalbumin and β–lactoglobulin, can be cross–linked by TGase (Tzikas and Ambrosiadis 2005). TGase catalyzes the acyl transfer reaction between the carboxyamide groups of

peptide–bound glutamine residue and a variety of primary amines (e.g. protein/peptide–bound lysine) (Ohtsuka et al. 2000) (Figure 3). This reaction leads to the formation of intra– and inter–molecular covalent bonds that cross–link different protein molecules together to make a big protein complex that enhances the product organoleptic properties. Generally, TGase can be extracted from different sources such as animal and microorganism. TGase extracted from pig had been studied in several food systems (Folk 1983; Nio et al. 1985; Ohtsuka et al. 2000) and so did those from microorganism (Ohtsuka et al. 2000; Shand et al. 2008; Sun and Arntfield 2011a). Currently, a microorganism–derived TGase has been commercialized by Ajinomoto and Amano Pharmaceutical.

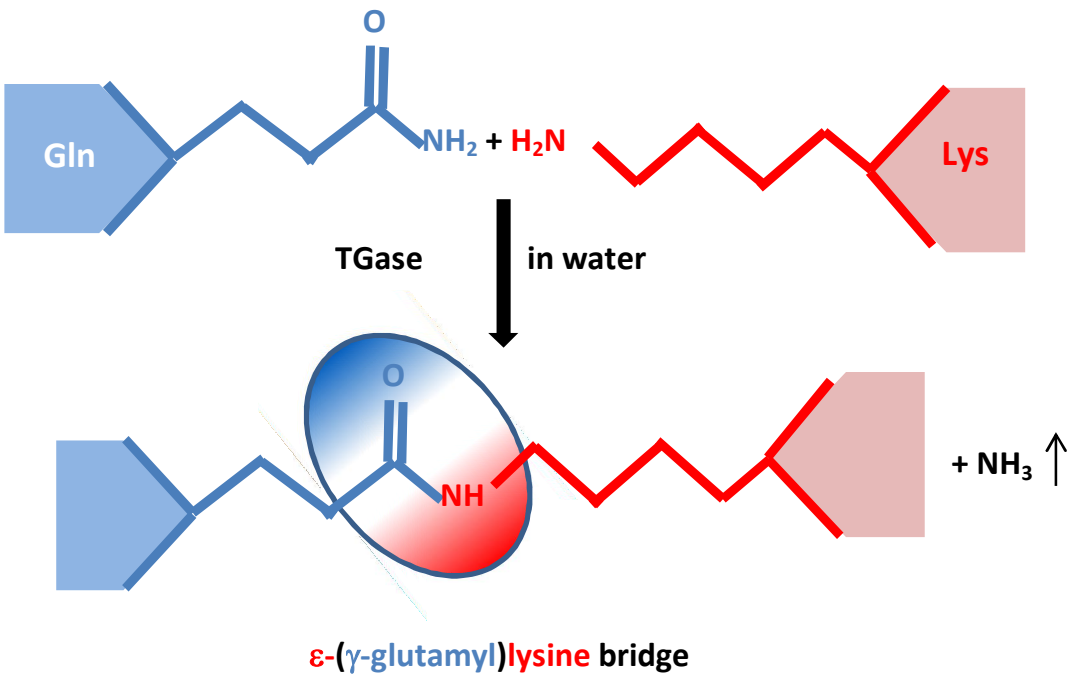


Figure 3. Schematic diagram of transglutaminase catalyzed reaction.
Redraw from http://www.bioeng.cstm.kyushu–u.ac.jp/.

The ability of TGase to modify the functional properties of food proteins, both animal based– and plant based proteins, has been extensively reported (Lorenzen 2000;

Kuraishi et al. 2001; Gerrard 2002; Lauber et al. 2003; Rodriguez–Nogales 2005; Tang and Ma 2007; Bruno et al. 2008; Ribotta et al. 2008; Ali et al. 2010, Sun and Arntfield 2011a). Folk (1983) proved the possibility of guinea pig liver TGase to promote modification of functional properties in milk casein and soybean globulins. Motoki and seguro (1998) reported TGase works on different system including whey protein, and actomyosin from beef, pork, chicken or fish. TGase functioning on other systems was also reported such as oat globulin (Siu et al. 2002), pea protein (Shand et al. 2008), phaseolus, and cow pea (Ahmed et al. 2011), pigeon pea (Ali et al. 2010; Ahmed et al. 2011), and peanut protein (Hu et al. 2011). Not only working on homogenous protein systems, TGase has been found to be able to cross–link proteins from different sources, e.g. peanut protein with fish protein (Hu et al. 2011) and myofibrillar with soy protein (Ramirez–Suarez and Xiong 2003).

TGase treatment was reported to improve functionalities of native protein systems including viscosity, yield stress, gelling capacity, water–holding capacity, thermal stability, emulsifying ability and foaming ability. Effect of TGase on oat globulins had been studied by Siu et al. (2002). These authors reported that as TGase incubation progressed, changes in flow properties of oat globulin dispersions were observed, indicating enhanced pseudoplasticity and increased viscosity and yield stress. Motoki and Seguro (1998) reported the improvement in gelling capacity of highly concentrated solution of whey protein and actomyosin from beef, pork, chicken or fish with the use of TGase. Subsequently, increase in solubility, water–holding capacity and thermal stability of food protein was demonstrated (Motoki and Seguro 1998). Nonaka et al. (1994) successfully used TGase to improve the gelation of soy protein isolate up to

two time stronger compare to the control. The gel hardness increased as the TGase concentration increased within the range tested meanwhile, cohesiveness reached max value at TGase concentration of 1 μg/g of protein (Nonaka et al. 1994). Chanyongvorakul et al. (1995) studied gelation of 11S globulins isolated from soy glycinin and broad bean legumin with TGase and found that gels produced by TGase treated samples formed superior networks compared to heat induced ones. Effect of TGase on rapeseed protein was studied by Hyun and Kang (1999). The authors found that TGase successfully induced the gelation of rapeseed protein and gelation increased linearly to the treatment time up to 90 mins. Optimal conditions of 45^0C and pH 7.0 were identified for TGase treated rapeseed protein gelation (Hyun and Kang 1999).

Pea protein isolate treated with microbial TGase was reported to improve gel strength and elasticity that was similar to those of commercial soy protein isolate's gel (Shand et al. 2008). Sun and Arntfield (2011a) found that gel strength of pea protein isolate treated with microbial TGase was stronger than that of soy protein isolated treated with microbial TGase. Motoki and Seguro (1998) found that proteins in oil–in–water type emulsions could also be gelled. Emulsifying and foaming properties of TGase treated proteins were also reported to improve compared to native protein by different authors (Ali et al. 2010; Ahmed et al. 2011; Hu et al 2011). Ahmed et al. (2011) studied effect of cross–linking of different protein isolates from legumes by TGase treatment on functional properties at different pH levels. The protein isolates that were investigated were from phaseolus (*Phaseolus vulgaris*), pigeon pea (*Cajanus cajan*) and cowpea (*Vigna unguiculata*).The authors found that emulsifying and foaming properties of the TGase treated protein isolates were greatly improved at all pH levels tested, except at pH 4,

compared to the native protein isolates. The solubility of treated samples was also greatly improved at pH 2 and pH values from 8 to 12 whereas, at pH 4 and 6, solubility of some legumes protein was slightly lower (Ahmed et al. 2011). Better heat stability, i.e. less turbidity on heating to higher temperature, was another finding in their work (Ahmed et al. 2011). Emulsifying and foaming properties of the TGase treated pigeon pea and hyacinth bean proteins were greatly improved at a wide range of pH level compared to native proteins (Ali et al. 2010). TGase can also cross–link proteins from different sources and improve the mixture emulsifying properties as indicated in Hu et al. (2011). The study showed that TGase cross–linking could improve the emulsifying properties of peanut protein isolate, peanut protein isolate and fish (*Decapterus maruadsi*) protein hydrolysate (DPH) system, and pea protein isolate hydrolysis (PPIH) and DPH system. The authors also suggested that proteolysis followed by TGase treatment crosslinking would further improve emulsifying properties (Hu et al. 2011).

However, the introduction of cross–links between proteins might affect the nutritional quality of final product (Gerrard 2002). The formation of unnatural covalent cross–linking of amino acids, either intra– or inter–molecular, may decrease the digestibility and biological availability of essential amino acids that are involved in cross–linking (Erbersdobler 1989). Volken de Souza et al. (2009b) found that cross–linking process significantly improved the true digestibility for soy protein but decreased that for meat protein. Other factors such as biological value, net protein utilization, net protein ratio, and protein retention efficiency values for both meat and soy proteins were not affected (Volken de Souza et al. 2009b). They suggested that the use of TGase for the reticulation of isolated soy proteins can improve their nutritional quality (Volken de

Souza et al. 2009b). Impact of TGase treatment on other proteins, i.e. milk protein and wheat protein, was also studied by these authors. They observed that protein efficiency ratio, food efficiency ratio, food transformation index, apparent nitrogen digestibility, true digestibility, biological value, net protein utilization, net protein ratio, and protein retention efficiency were not affected (Volken de Souza et al. 2009a). The authors suggested that the use of microbial TGase does not affect the nutritional quality of milk and wheat proteins, while improving their physicochemical properties (Volken de Souze et al. 2009a).

Since pea protein is rich in lysine and glutamine, we expect the TGase will work well on pea protein, leading to the improvement in its functionalities, especially gelling capacity, and produce cross linked proteins with foaming, emulsifying and gelling properties similar to egg proteins.

Foams and emulsions are both multiple–phase systems. Foams are dispersion of gas(es) in liquid(s) whereas emulsions are disperse systems of one or more immiscible liquids (Belitz et al. 2009). Proteins can form foams and stabilize foams and emulsions systems due to its amphipathic nature (Belitz et al. 2009). Key factors determining such abilities of proteins are the rate at which proteins diffuse into the interface of immiscible phases and the deformability of proteins conformation under influences of interfacial tension, i.e. surface denaturation (Belitz et al. 2009). At interface, protein molecules change from normal conformation to train–loop–tail conformation (Figure 4). “Trains” are segments of a flexible polymer lying in direct contact with the surface, whereas “loops” and “tails” dangle into the bulk phase(s) (Sjoblom 1991). The amino acid composition and amino acid sequencing in a protein molecule determine the ability of

that protein to perform as a foaming and emulsifying agent. Amino acids with aliphatic and aromatic side chains are hydrophobic, and hence they exhibit limited solubility in water (Fennema 1997). Those amino acids are present dominantly in train segments, giving them a fairly hydrophobic nature. As a consequence, the trains tend to stay at the interface. In contrast, loops and tails have more polar amino acids, therefore, they are hydrophilic in nature and stay in the bulk aqueous phase. The greater the proportion of polypeptide segments in a train configuration, the stronger is the binding and the lower is the interfacial tension (Fennema 1997).

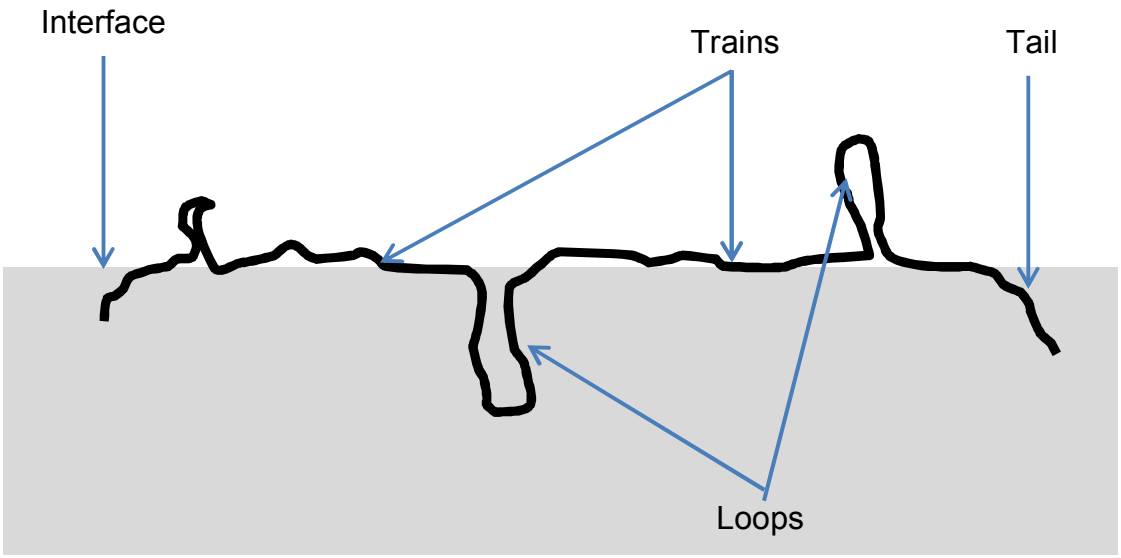


Figure 4. Schematic diagram of protein conformation at hydrophilic–lipophilic interface. Redraw from Damodaran (1990).

Current testing methods for both emulsifying and foaming effect of pea protein employed a high speed blender to create foam and emulsion systems. Proteins with a better foaming effect will form a larger volume of foam and this foam system will be stable for longer time under the same testing conditions of mixing speed, mixing time, temperature, and protein concentration. Similarly, a protein with a better emulsifying

effect can incorporate a larger amount of oil into an emulsion system and this system is stable for a longer period of time under the same testing conditions. A protein with ideal qualities as an emulsifier and foaming agent would have a relatively low molecular weight, a balanced amino acid composition in term of charged, polar and nonpolar residues, good water solubility, well–developed surface hydrophobicity, and a relative stable conformation (Belitz et al. 2009).

5. CAKE AND COOKIE SYSTEMS

Quality of bakery products such as cakes and cookies mainly rely on foaming and emulsifying properties of their raw ingredients, respectively. Among all raw ingredients, egg protein is responsible for such properties and therefore is considered a key ingredient in bakery products. Cakes are among the most popular egg–containing products in the confectionery category. Cakes are well known for their sponge–like texture, which is mainly a result of foaming capacity and coagulating capacity of egg proteins. Egg protein facilitates air incorporation into a batter during whipping (Bennion and Bamford 1997) while coagulating capacity, when heated, helps to set the crumb of the cake (Amendola and Rees 2003). In regular cake formulas, fresh whole egg is usually used and its percentage is usually counted for about 70 % to 97.8% (flour based) (Bennion and Bamford 1997; Manley 1998). In formulas using cake mix, the fresh egg to cake mix ratio is reduced and stays in a range of 20% to 30% (cake mix based) due to the fact that cake mix is the mixture of several powder ingredients, i.e. flour, sugar, baking powder, flavor, and color. If based on wheat flour only, this ratio is in agreement with the above range.

Generally, cakes can be classified into one of the three groups: foam cakes, batter cakes, or chiffon cakes. Based on the forms of leavening that are used, batter cakes can be divided into two subdivisions: layer cake and pound cake. Layer cake contains chemical leavening agents in the formula and pound cakes are leavened only by air incorporated and entrapped during the mixing process (Zhou 2010). High ratio cakes are those in which the weight of the sugar is equal to or greater than that of the flour (Pyler 1988; Zelch 2001). In these formulas, sugar has an important role in delaying starch gelatinization during cake baking so that air bubbles can be properly expanded by carbon dioxide and water vapor before the cake sets (Yamazaki and Kissell 1978).

Currently, commercial cake mix is very common in US market place. Typically, in order to make a cake at home, consumers just need to buy a cake mix box, along with an appropriate amount of oil, water and fresh egg as indicated on the box to complete the preparation. The two types of cake mixes available use either whole egg or just egg white portions. Both high ratio and low ratio formula cake mixes have been developed. Betty Crocker, Duncan Hines, Pillsbury, Martha Whites, Krusteaz, and Jiffy are common producers of cake mix found in the local market. On a typical commercial cake mix formula, egg usually accounts for approximately 25% of the formula based on cake mix weight.

Several cake formulas for evaluation of flour performance have been developed (Yamazaki and Kissell 1998; Approved Method 10–90 AACCI 2000; American Institute of Baking; Zhou 2010). Those formulas were developed based on the combination of different individual ingredients and were used to test the effect of different ingredients or

their substitutes on the overall quality of the cake. Cake formulas can also be found in many different cookbooks.

In cookie system, spray–dried egg is more commonly used than fresh whole egg due to the difficulties of cracking and subsequent handling of eggs (Manley 1998). Cookies with only egg whites produce crispier than chewy cookies as compared to formulas using whole egg (Amendola and Rees 2003). As one of the excellent emulsifiers (Bennion and Bamford 1997), egg yolk is also used in cookie making in order to increase the effectiveness of the fat in cookie dough. If dough contains significant amount of egg, the final products will have a soft cake quality (Amendola and Rees 2003). The average amount of dried egg used in cookie formulas is approximately 1% (flour based) (Manley 1998).

Cookie formulas greatly depend on geographic region, types of cookies and manufacturers. In US, different producers provide different formulas to target nutritional and health benefit claims, i.e. high dietary fiber, high protein or less fat. American Association of Cereal Chemists International (AACCI) provides a general formula for flour testing purpose in cookies (Approved method 10 –53.01 AACCI 2000). American Institute of Baking (AIB) also provides another formula for cookies. Different cookie formulas can also be found in cook books.

No study of the effect of pea protein on cake and cookie systems has been found in literature, but there are several studies relating to using other vegetable protein ingredients in making cakes and cookies. Singh and Mohamed (2005) studied the influence of gluten –soy protein blends on the cookie system and the result showed that replacing up to 15% of total carbohydrates in cookie formulas by protein blends did not

significantly affect the width and height of the cookies (Singh and Mohamed 2005). Although the replacement affected cookies' diameter, this study did imply that vegetable protein can be used as part of the cookie formulas. Meanwhile, Gomez et al. (2008) studied the effect of chickpea flour on cake quality. The authors reported that replacing wheat flour with chickpea flour induced an increase in the initial firmness of the cakes but diminished their volume, symmetry, chroma, crust, crumb, cohesiveness and resilience. This study aimed to replace wheat flour with chickpea flour instead of studying functionalities of chickpea flour as an egg replacer in cake systems, thus the comparison between egg and no egg was not investigated.

6. SENSORY AS AN ANALYTICAL METHOD

Consumer sensory test is a scientific method used to provide manufacturers consumers' opinions and reactions about their products through a series of steps including collecting data, analyzing, interpreting, and providing usable outputs. It is defined as "a scientific discipline used to evoke, measure, analyze, and interpret reactions to those characteristics of foods and materials as they are perceived by senses of sight, smell, taste, touch, and hearing" (IFT sensory evaluation division 1975). Products are evaluated on the basis of appearance, taste, smell, touch, and hearing (ASTM 1979). Consumer sensory is different from market research testing in the way that it is generally conducted with coded, unbranded products, whereas the latter is most frequently done with branded products (van Trijp and Schifferstein 1995). There are two approaches to consumer sensory tests, which are the measurement of preference and the measurement of acceptance (Jellinek 1964).

Preference tests measure the appeal of one food or food product over another (Stone and Sidel 1993) and are useful when one product is compared directly against another (Resurreccion 1998). The consumer acceptance test gives an estimate of product acceptance based on the sensory properties. The results of the test provide an indication of product acceptance without the effect of the other factors, which can enhance or reduce its acceptance (Resurreccion 1998). In consumer sensory tests, many methods are described in literature but typically they all belong to three basic types which include (1) the paired preference, (2) ranking, and (3) rating tests (Resurreccion 1998). Of all methods, the rating test provides the most information as it gives direct measure of the magnitude of liking. The 9–point hedonic scale is a rating scale that has been used for many years in sensory evaluation in the food industry and has been validated in the scientific literature (Stone and Sidel 1993). 3–, 5–, and 7–point hedonic scales are also used but were found to be appropriate for 3–, 4–, and 5–year–old children, respectively (Chen et al. 1996).

Consumer sensory acceptance tests have been used quite frequently in cake quality determination, especially in the cases where egg was replaced by egg replacers in cake formulas. Abu–Ghoush et al. (2008) used a 9–point hedonic rating test to test consumer acceptance on angel food cake using different ingredients as an egg replacer, e.g. Cryogel gelatin, whey protein isolate, whey protein concentrate, and collagen. Recently, the same sensory method was used to test consumer acceptance on yellow cake with egg component was substituted by different ingredients at the levels of 50% and 100% (Kohrs et al. 2010). However, no literature is found on using pea protein isolate as an egg replacer in cake formula.

PRELIMINARY INVESTIGATION

1. STUDY ON DIFFERENT RAW MATERIALS

Preliminary results indicated that yellow pea had a better protein yield compared to that of green pea at the same extraction conditions, i.e. 19.6% vs. 15.1%, respectively, thus yellow pea was chosen as pea source for this project. Four different types of yellow peas including non–roasted split yellow pea, roasted split yellow pea, non–roasted whole yellow pea, and roasted whole yellow pea were also studied on preliminary and protein yield results showed that non–roasted split yellow pea had the highest yield at all pH levels. Therefore, non–roasted split yellow pea was chosen (Table 3).

Table 3. Protein yields from different pea sources* extracted at different pH values.

Extracting pH	Yield, % PE/PF, w/w			
	NR split	R Split	NR whole	R whole
10	16.35	9.63	16.09	2.94
9	15.64	8.90	14.98	2.36
8	14.15	8.04	13.72	1.98
7	12.69	7.06	12.29	1.77
6	4.15	1.06	4.42	1.05
5	0.65	0.07	0.69	0.18

* NR: non–roasted, R: roasted, PE: protein extracts, PF: pea flour. All samples were yellow peas.

2. PRELIMINARY CAKE SENSORY EVALUATION

Preliminary sensory evaluation on cake height indicated a poor performance of both spray–dried control PPI and spray–dried TGase treated PPI (Table 4), thus these two protein extracts were eliminated from preliminary sensory evaluation.

Table 4. Cake height measurements of different cake formulas* using approved method 10–91 (AACCI 2000).

Formula	Cake heights, mm**				
	A	B	C	D	E
TS	20	12	12	12	22
CS	22	10	10	11	28
TF	23	29	31	28	22
Egg white	22	37	44	38	26
CF	26	26	28	25	24
Cake mix	22	29	32	29	22

* TS: spray–dried TGase treated pea protein isolate, CS: spray–dried control pea protein isolate, TF: freeze–dried TGase treated pea protein isolate, CF: freeze–dried control pea protein isolate.

** A and E: edge positions, B and D: 4 cm from edges, C: center position (refer to Figure 7 in Materials and Methods section).

Preliminary sensory conducted on 30 panelists for cakes containing different proteins including freeze–dried control pea protein isolate, egg white protein, TGase treated freeze–dried pea protein isolate, and cake mix as a control, suggested that egg white formula had the best texture, followed by control PPI, TGase treated PPI, and cake mix formulas (Table 5).

Table 5. Preliminary acceptability of different cake formulas* using a 9 point hedonic scale.

Formula	Appearance	Flavor	Texture	Overall
Control PPI	5.6	5.3	4.7	5.2
Egg white	6.4	6.5	6.5	6.5
TGase treated PPI	5.7	4.1	4.6	4.3
Cake mix	4.9	4.9	4.0	4.7

* PPI: pea protein isolate, results was an average score of 30 replicates on a 9 point scale.

Off–flavor caused a poor score on TGase treated PPI formula and consequently its lowest overall acceptability. However, the results showed that all samples containing proteins were better than cake mix only formula in both texture and appearance (Table 5). Therefore, cake mix formula was eliminated from the final sensory evaluation. This decision was also applied for cookie sensory, meaning only freeze–dried protein extracts and egg formulas were used to test consumer acceptability.

Furthermore, cake–mix based cakes did not have a good cake crumb quality, suggesting a formula developed from individual ingredients was needed.

MATERIALS AND METHODS

1. MATERIALS

1.1. Raw material

Yellow split pea samples were purchased from Specialty Commodities, Inc (Fargo, ND). The yellow pea samples were a mixture of different varieties. Yellow split pea was chosen as pea source for this project because it had the best yield and other organoleptic parameters compared to green pea or whole peas based on preliminary research. Furthermore, yellow pea is more readily available than other peas, i.e. 307,826 MT of yellow pea compared to 208,784 MT of green pea in 2008 (US Dry Pea and Lentil Council http://www.pea–lentil.com/).

Yellow split peas were ground into fine particles using a hammer mill (Fitz Mill, Model: DASO 6, series no. 11984 – The FitzPatrick Company, 832 Industrial Drive, Elmhurst, Illinois 60126, U.S.A.). The pea flour had at least 99% pass through sieve no. 20 (0.841 mm). Both feed and grinding speeds were set to optimum condition for pulse. The hammer mill configuration employed a blunt-face hammer operated with a tip speed of approximately 5,200 m/min (7,200 rpm), feed rate of 2.5 kg/min (20 rpm), and a screen (code 1532 0050) opening of 0.127 cm.

The rotor speed can be converted to velocity of the hammers. The velocity of the hammers is critical for proper size reduction. Tip speed is the speed of the hammer at it's tip or edge furthest away from the rotor (http://www.feedmachinery.com/glossary/hammer_mill.php), and is calculated using the equation:

$$\text{Meter per minute} = \frac{\pi D \times rpm}{100\ (\frac{cm}{m})}$$

$$= \frac{3.14 \times 23\ (cm) \times 7200}{100\ (\frac{cm}{m})} \approx 5{,}200\ \text{m/min}$$

In which: $\pi = 3.14$;

D: diameter in cm;

rpm: rotor revolutions per minutes.

Pea flour was cooled after milling to room temperature, and kept in plastic bag in bulk, i.e. 50 lbs/bag, and re–conditioned in a cool room (18–25^{0}C) for at least 48 hrs before being used in the extraction process.

Roquette, Nutralys F85M, a commercial PPI, used in this research as a benchmark.

1.2. Transglutaminase

Transglutaminase (TGase, E.C. 2.3.2.13) used in this project was purchased from Ajinomoto Food Ingredients, LLC (Chicago, IL) in 1kg foil pouches. Among different products in the Ajinomoto TGase line, Activa–TI was chosen due to the fact that this enzyme has a high enzymatic activity, i.e. higher than 100 U/g, and contains no lipid. According to the manufacturer, Activa–TI consisted of 99% maltodextrin and 1% enzyme on a mass basis. Each gram of this product provides 100 units of enzyme activity and the product is stable for 24 months under normal conditions, i.e. 70^{0}F or less, if stored unopened. After being opened, the TGase was divided into smaller 100g sachets, vacuum sealed, and stored at 4–10^{0}C in the dark prior to use.

1.3. Chemical and other minor materials

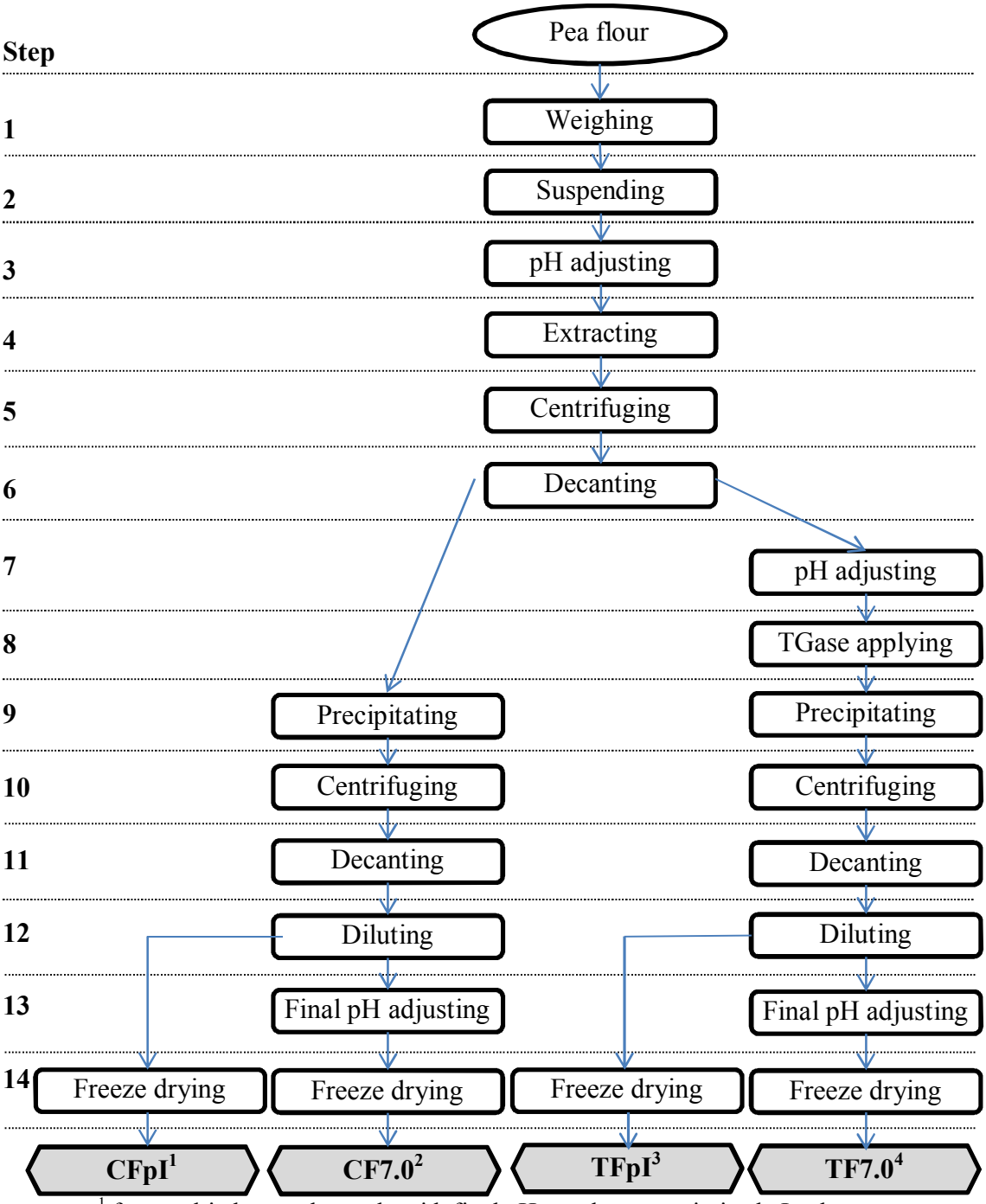
Hydrochloric acid (HCl, min 36.5%) was from J. T. Baker and anhydrous sodium hydroxide (NaOH anhydrous powder) was from VWR. Hydrochloric acid was diluted in de–ionized distilled water to make a 10% solution whereas anhydrous sodium hydroxide was dissolved in de–ionized distilled water to make a 2N solution.

Oil used in this project was soybean oil, i.e. Wesson brand, purchased from Hornbachers grocery store (Fargo, ND). Other ingredients used in baking such as egg, sodium chloride, all–purpose wheat flour (Dakota Maid brand), cake flour (Softasilk brand), shortening (Crisco), butter (Cass–Clay band), brown sugar, granular sugar, and baking powder were also purchased from Hornbachers. Vanilla powder was purchased from Cake & Candy World (Fargo, ND). Egg albumin powder was from Fischer Scientific Company.

2. METHODS

2.1. Extracting methods

Generally, there were two approaches used in extracting pea protein from yellow split pea flour. The schematic diagram for the first extraction method is summarized in Figure 5. Briefly, this approach included two different procedures, one for extracting control samples where no enzyme treatment was used and another with the use of enzyme to produce enzyme treated samples. Each sample had two versions, one with no final pH adjusted, i.e. retained the pH most closely to the pI, and the other with the final pH adjusted to 7.0. Only the no final pH adjusted method was used to evaluate optimum extraction conditions. However, extracts from both methods were used in model tests.



[1] freeze–dried control sample with final pH equal to an optimized pI value
[2] freeze–dried control sample with final pH of 7.0
[3] freeze–dried TGase–treated sample with final pH equal to an optimized pI value
[4] freeze–dried TGase–treated sample with final pH of 7.0

Figure 5. Extraction protocol for pea protein using the freeze drying method.

Much of the research focused on extraction parameters which ended with drying using a freeze drier. Optimizing the extraction pH was completed first, followed by optimizing precipitating pH (i.e. pI), flour–to–water ratio, extraction time, and TGase treatment. Protein yield was used as an indicator for optimum extraction pH, optimum precipitating pH, flour–to– water ratio, and extraction time. Viscosity from RVA, molecular weight profile from SDS gel, and functionalities were used as indicators for optimum pH for TGase treatment.

Extraction pH (Step 3 in Figure 5): To determine the effect of pH on protein extraction, the pH of the extracting solution was adjusted to several pH values between 7.0 and 10.0. The flour–to–water ratio (1:6), precipitating pH of 4.5, and extraction time of 30 mins were held constant. The optimized extraction pH value determined in this experiment was then used for optimizing other parameters.

Precipitating pH (Step 9 in Figure 5): To determine the effect of pH on protein precipitation, the pH of the supernatant was initially adjusted to several pH values between 5.0 and 3.7. The narrower range was then targeted and second pH adjustment was done in this new pH range, i.e. 4.5 to 4.0. The flour–to–water ratio (1:6), extraction pH value determined in the previous step, and extraction time of 30 mins were held constant. The optimized precipitating pH value determined in this experiment was then used for the following optimization steps.

Flour–to–water ratio (Step 2 in Figure 5): To determine the effect of flour–to–water ratio on protein extraction, the flour–to–water ratio was adjusted to six different values including 1:4, 1:6, 1:8, 1:9, 1:10, and 1:12. Extraction pH and precipitating pH

values determined in the previous steps, and extraction time of 30 mins were held constant.

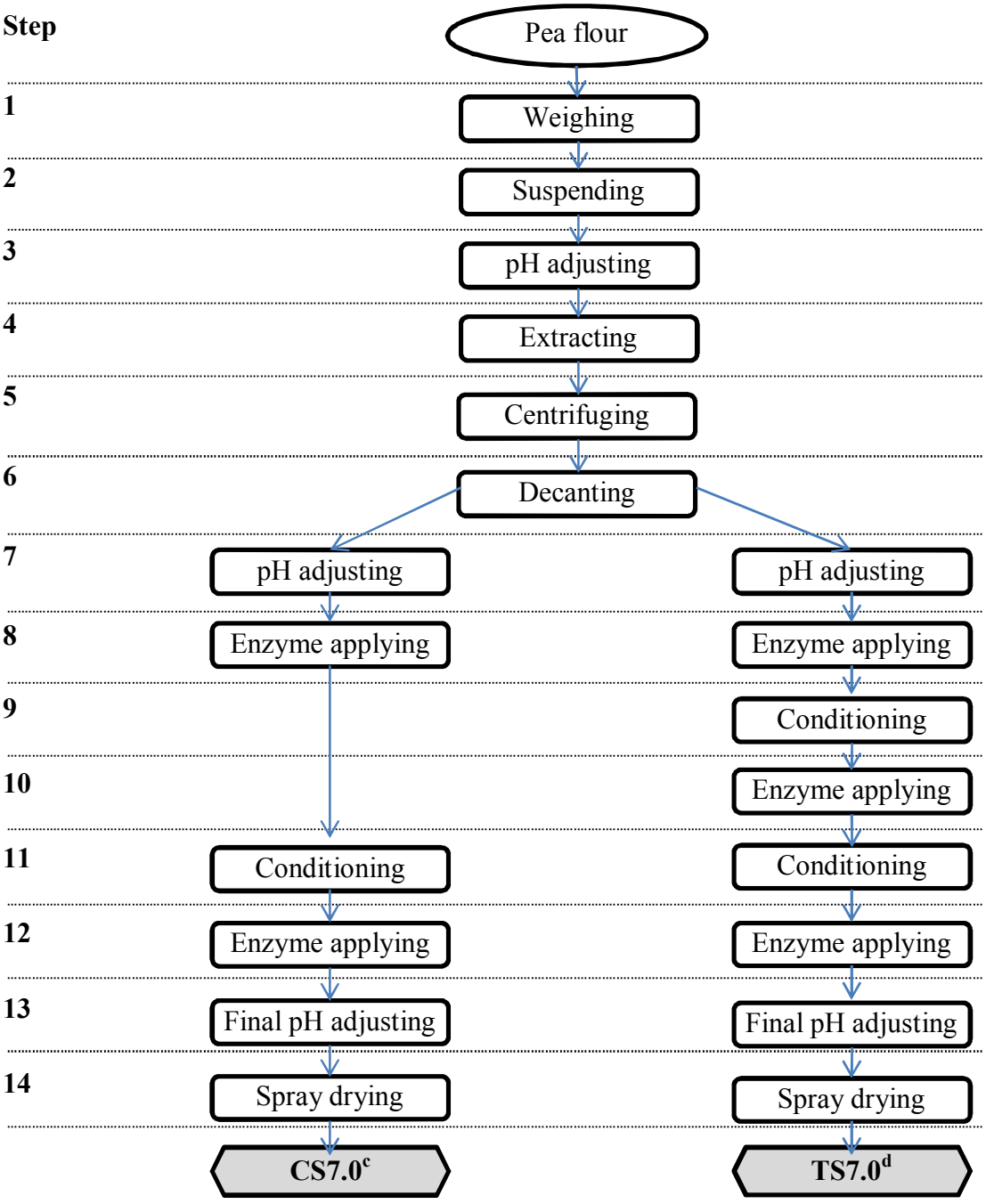
Extraction time (Step 4 in Figure 5): To evaluate the effect of extraction time on protein extraction, the extraction time was adjusted to different values between 15 to 45 mins. Extraction pH, precipitating pH values, and flour–to–water ratio, which were determined from the previous steps, were held constant.

TGase treatment (Step 7 in Figure 5): To study the effect of pH values on TGase catalyzed reaction in pea protein slurry, the TGase was applied at different pH values between 7.0 and optimized pI value. Optimum extraction pH, precipitating pH values, flour–to–water ratio, and extraction time obtained from previous steps were used to obtain the protein for cross–linking.

In general, the first extraction approach consisted of 14 steps (Figure 5). Yellow pea flour was first weighed and suspended in distilled water, i.e. approximately 25^0C, at an optimum flour–to–water ratio. pH of the slurry was then adjusted to an optimum value using sodium hydroxide solution (2 N) and the slurry was stirred at low speed using a magnetic stirrer for an optimum amount of time at room temperature (approx. 25^0C). Centrifugation (22,000 x g for 3 mins) was applied and the supernatant retained. To prepare control samples, the supernatant was adjusted to an optimum pI using hydrochloric acid solution (2 N) followed by a second centrifugation (23,000 x g for 5 mins). The supernatant was discarded and the pellet diluted with minimal amount of distilled water. Pea protein slurries were then either directly freeze–dried to make control samples with final pH equal to pI, i.e. CFpI, or pH adjusted with sodium hydroxide solution (2 N) to make control samples with final pH of 7.0, i.e. CF7.0 (Figure 5).

TGase–treated samples were prepared by first adjusting pH of the supernatant to an optimum value for TGase, followed by the addition of TGase solution (100 U/mL) at a rate of 1.7 U/g pea flour, and incubation (40^0C in 30 mins) in water bath. The pH of the slurry was adjusted to an optimum value after incubation was completed and centrifuged (23,000 x g for 5 mins) to separate pea protein. Pellet was then collected and minimally diluted with distilled water. Again, the protein slurries were either pH adjusted to 7.0 before freeze drying to make TGase–treated samples with final pH of 7.0, i.e. TF7.0, or directly freeze–dried to make TGase–treated samples with a final pH equal to the pI, i.e. TFpI (Figure 5).

The schematic diagram for the second approach was summarized in Figure 6. In general, optimum extraction conditions described above were followed in the second approach. However, the second approach incorporated several steps involving enzyme treatments. In contrast to the first approach, there was no protein precipitation step in the second approach as the whole supernatant was used in spray drying step. Instead, the second approach used enzymes to reduce non–protein components. Three different enzymes were used in the second approach included α–Amylase, amyloglucosidase (both from total starch assay kit from Megazyme) and TGase (Ajinomoto Foods Ingredients, LLC – USA). The use of α–Amylase and amyloglucosidase in this approach was to reduce soluble starches in the supernatant and thus reduce starch effects on pea protein functionalities.



[c] spray–dried control sample with final pH of 7.0
[d] spray–dried TGase–treated sample with final pH of 7.0

Figure 6. Extraction protocol for pea protein using the spray drying method.

Briefly, the second approach consisted of 14 steps (Figure 6). Yellow pea flour was first weighed and suspended in distilled water, i.e. approximately 25^0C, at an optimum flour–to–water ratio. pH of the slurry was then adjusted to an optimum value

using sodium hydroxide solution (2 N) and the slurry was stirred at low speed on magnetic stirrer for an optimum amount of time at room temperature. Centrifugation (22,000 x g for 3 mins) was applied and the supernatant collected.

To prepare control samples, the supernatant was adjusted to pH 6.5 using hydrochloric acid solution (2 N), followed by an addition of α–Amylase solution (3,000 IU/mL) at a rate of 17 U/g pea flour and incubation at room temperature for 30 mins. The pH of the supernatant was adjusted to 4.5 to inhibit α–Amylase and then amyloglucosidase (3,300 IU/mL) was added at a rate of 1.7 IU/g pea flour, followed by incubation at 50^0C for 30 mins. The whole supernatant was adjusted to pH 7.0 and then spray–dried (input temperature 180 – 200^0C, output temperature 90 – 100^0C) to make control samples, i.e. CS7.0 (Figure 6).

To prepare TGase–treated samples, the same α–Amylase treatment process was first applied as conducted in preparing control samples. The pH of the supernatant was then adjusted to 6.0 after incubation and TGase solution (100 U/mL) was added at a rate of 1.7 U/g pea flour, followed by incubation at 40^0C for 30 mins. When TGase treatment completed, amyloglucosidase treatment was carried out with the same settings as in preparing control samples, i.e. enzyme was added at a rate of 1.7 IU/g pea flour, followed by incubation at 50^0C for 30 mins. Finally, the pH was adjusted to 7.0 and the supernatant was spray–dried according to previous described methods to give TGase–treated samples (Figure 6).

The biggest difference between the two approaches was the method of dehydration. The first approach used freeze drying method whereas the second approach

used spray drying method. Thus, the spray drying method dried all components obtained during extraction.

2.2. Analytical methods

2.2.1. Size distribution

Particle size of milled yellow split pea flour was evaluated in the protein extraction process. The interest in particle size relates to the efficiency of the extraction. The particle size could be a method to improve efficiency if selected properly.

Particle size was done on a RO–TAP testing sieve shaker model B (C.E. TYLER, U.S.A). The sieves used were no. 20, no. 80 and no. 100. Particles left on sieve no. 20 (0.841 mm) were considered extra–large particles; particles pass through sieve no. 20 but were retained on sieve no. 80 (0.177 mm) were considered large particles; between sieve no. 80 and sieve no. 100 (0.149 mm) were classified as medium size particles; and particles through sieve no. 100 were fine particles.

Sieving was done in controlled environments where air temperature and relative humidity were kept lower than 25^0C and 80%, respectively. Under these conditions, flour caking was prevented and thus minimizing inaccuracy due to large clumps. Approximately 50 g of yellow split pea flour was used for each size distribution test and the shaking time was set at 3 mins. After sieving, pea flour portion on each sieve was weighed and data recorded. The flour portion on the last tray was also weighed and recorded. Total weight was the added value of all portions. Weighed portions were then converted into percentage to indicate size distribution.

2.2.2. Moisture content

Moisture content of pea flours, pea protein concentrates, and pea protein isolates was determined using conventional air oven method (Approved Method 44–15A AACCI 2000). One stage moisture determination was chosen. In this method, 2 to 3 g of ground sample were heated in an air oven to 130^0C for 1 h and then the weight of the residue was calculated. Weight of residue was considered weight of the total solids and weight loss after drying was considered moisture weight. This was an indirect method of moisture weight determination.

Moisture content was then calculated using the following equation

$$\% \text{ moisture} = \frac{A}{B} x100$$

In which A = weight loss in grams, B = original weight of sample.

2.2.3. Total starch

Total starch determination was carried out using the Megazyme Total Starch Assay procedure, which was based on two Approved Methods (996.11 AOAC 1997 and 76–13 AACC 2000) with improvements. All chemicals and enzymes were provided in the Megazyme assay kit. Two different methods were used to determine total starch in different pea protein samples.

To determine total starch in freeze–dried pea protein isolates, the method (a), i.e. "Determination of starch in cereal and food products not containing resistant starch, D–glucose and/or maltodextrins" was used. This procedure consists of two separate steps: sample preparation and sample assay. Pea protein sample was ground to pass a 0.5 mm

screen, and then approximately 100 mg added into a glass test tube (16 x 120 mm). An aqueous ethanol (80% v/v, 0.2 mL) was added, mixed on a vortex mixer for several seconds and then 3 mL of an α–Amylase solution, i.e. bottle 1 diluted 1:30 in sodium acetate buffer (100 mM, pH 5.0), immediately added. Incubation started when tubes were placed in a boiling water bath. Samples were vigorously stirred at 2, 4 and 6 mins. After 6 mins, samples were cooled in 50^0C water bath before amyloglucosidase (3,300 U/mL, 0.1 mL) was added to the mixture. Incubation at 50^0C continued for 30 mins. The whole crude mixture was quantitatively transferred to a 100 mL volumetric flask and diluted with distilled water to make 100 mL mixture. Centrifugation at 3,000 rpm (1,800 x g) for 10 mins was used to separate residues. Clear filtrate was used for the assay and was analyzed in duplicate. In the assay step, 0.1 mL of filtrate was transferred to the glass test tubes (16 x 100 mm). Glucose Determination Reagent (GOPOD Reagent, 3 mL) was added and the mixture incubated at 50^0C for 20 mins. After incubation, sample absorbance at 510 nm was measured against a reagent blank. D–glucose controls were also prepared by combining 0.1 mL of D–glucose standard solution at concentration of 1 mg/1 mL (provided) and 3.0 mL of GOPOD Reagent.

To determine total starch in spray–dried pea protein concentrates, the method (e), i.e. "Determination of starch in samples which also contain D–glucose and/or maltodextrins" was used. The sample preparation was slightly different from that of freeze–dried sample but the assay was identical. Pea protein sample was ground to pass 0.5 mm screen, and then approximately 100 mg added into a glass test tube (16 x 120 mm). An aqueous ethanol (80% v/v, 5 mL) was added, incubated at 80–85^0C for 5 mins. The content was mixed on vortex mixer for several seconds and another aqueous ethanol

(80% v/v, 5 mL) added. Samples were then centrifuged at approximately 3,000 rpm (1,800 x g) and supernatants discarded. A second extraction was done by re–suspending the pellet in aqueous ethanol (80% v/v, 10 mL), stirring on a vortex mixer and centrifuging (1,800 x g). The remaining steps were identical to those used for freeze–dried samples, i.e. starting by adding 3 mL of α–Amylase to the pellet to initiate the reaction.

Total starch contents were calculated using the formula.

$$\text{Starch, \% as is} = \text{Abs} \times \frac{F}{W} \times FV \times 0.9$$

Abs = absorbance (reaction) read against the reagent blank.

$F = \frac{100\ (\mu g\ of\ D\text{–}glucose)}{Abs\ for\ 100\ \mu g\ of\ glucose}$: conversion from Abs to µg.

W = the weight in milligrams ("as is" basis) of the pea protein flour analyzed.

FV = final volume, i.e. equals 100 mL.

2.2.4. Total ash determination

Total ash of pea flours, pea protein concentrates, and pea protein isolates were determined using Approved Method 08–03 (AACCI 2000). About 2 g of sample was weighed and placed into previously ignited, cooled and tared porcelain crucible. Crucibles were then placed in muffle furnace preheated to 600^0C. Incineration was allowed to occur at 600^0C for exactly 2 hrs. Crucibles were finally transferred directly to desiccator to cool then weighed after reaching room temperature (25^0C).

Total ash content was reported as the percent ash to first decimal place using the equation

$$\% \text{ ash} = \frac{\text{Weight of residue}}{\text{Sample weight}} \text{x} 100$$

2.2.5. Crude protein – combustion method

Protein content in pea flours, pea protein concentrates, and pea protein isolates were determined using a combustion method (LECO) (Approved Method 46–30 AACCI 2000). In this method, total nitrogen in samples was freed by pyrolysis and subsequent combustion at high temperature in pure oxygen (99.9%). All NO_x gas and N_2 then were converted to free nitrogen. Finally, total nitrogen was isolated and detected by a thermal conductivity detector. The output of this method was percentage of nitrogen present in the sample. The amount of flour required for each running was approx. 0.25 – 0.5 g and the crude protein percentage determined using the equation:

$$\text{Crude protein, \%} = \%\text{N} \times 6.25$$

2.2.6. Electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine protein components in pea flours, pea protein concentrates, pea protein isolates and other comparable materials, e.g. egg albumin, soy protein isolate, and commercial pea protein isolate. Regular SDS–PAGEs were selected to test the resolution of the method. The gel consists of two parts: separating gel, which was prepared from acrylamide/bis–acrylamide solution (30%T, 0.8%C), 1.5 M Tris–HCl, pH 8.8 buffer, 10% (w/v) SDS, and freshly prepared 10% (w/v) ammonium persulfate (APS) and stacking gel, which was prepared from above acrylamide/bis–acrylamide solution, 0.5 M

Tris–HCl, pH 6.8 buffer, 10% SDS, and 10% APS according to Khan and Nygard (2003). To initiate polymerization, N,N,N',N' tetramethylethylenediamine (TEMED) was added. Separating gel was prepared first, followed by the stacking gel. There were two separating gel concentrations to be tested, which were a typical concentration (12%) and a lower concentration (8%).

Protein samples were prepared in both non–reducing sample buffer (0.0625 M Tris–HCl, pH 6.8, 20% glycerol, 2% SDS, 0.0002% Pyronin–Y or Bromophenol blue) and reducing sample buffer (5% 2–mercaptoethanol or 1% dithiothreitol (DTT) in non–reducing sample buffer) using a suggested ratio in which 10 mg pea protein isolate was dissolved in 1 mL sample buffer and extracted at moderate temperature (i.e. 35^0C for non–reducing and 50^0C for reducing method) and periodically vortexed. After extraction was completed, samples were brought to 95^0C for 5 mins, cooled, centrifuged and loaded in wells on gel. A molecular weight standard (e.g. Bio–Rad SDS–PAGE MW standard) was also loaded in one well of each electrophoresis gel. The gels, after running, were stained by coomassie brilliant blue –G (CBB–G) and results read after staining and photographing.

2.2.7. Foaming capacity and stability

Foaming capacity and stability tests were done using methods currently used in our teaching lab. Briefly, pea protein samples were suspended in cold tap water (21 ± 1^0C) to make 0.5% w/v solution. This solution (200 mL) was transferred to an Oyster 12 speed blender (model 6843) and blended at high speed, grind mode for 1 minute. Foam was then transferred into graduated plastic centrifuge tube and initial volume was recorded as foaming capacity. The tubes were then covered by caps to eliminate effect of

environment, i.e. air flow, temperature change, and sat undisturbed at room temperature. Volume of foam at 5 and 30 mins were also recorded and used for calculating foaming stability.

Foaming capacity was calculated using the following formula

$$\text{Foaming capacity, \%} = \frac{\text{Foam volume at zero time}}{\text{Initial solution volume}} \text{x} 100$$

Foaming stability was calculated using the following formula

$$\text{Foaming stability, \%} = \frac{\text{Foam volume at 30 minute}}{\text{Foam volume at zero time}} \text{x} 100$$

2.2.8. Emulsion capacity and stability

Emulsion capacity and stability tests were accomplished using methods currently used in our teaching lab. In emulsion stability, pea protein samples were suspended in hot tap water (41 ± 1^0C) to make a 0.5% w/v solution. This pea protein solution (100 mL) was immediately transferred into an Oyster blender and mixed for 1 minute at high speed, i.e. blend mode. Then vegetable oil (Wesson brand, 75 mL) was added and mixed for an additional 3 mins. The emulsion system (14 mL) was transferred into each of the two 15 mL centrifuge tubes and was centrifuged at 1,500 rpm (405 x g) for 3 mins using an Allegra 2IR centrifuge (Beckman Coulter, U.S.A). Volumes of water released were recorded and used to calculate emulsion stability using the following formula:

$$\text{Emulsion stability, \%} = \frac{(14* - \text{Volume of water released})}{14*} \text{x} 100$$

* Milliliters of emulsion transferred into the centrifuge tube.

In emulsion capacity, 0.5% pea protein solutions were also prepared but with cold tap water (20–22^{0}C). This solution (40 mL) was transferred to a 250 mL glass beaker. The ULTRA–TURAX T25 BASIC S1 (IKA–WERKE, U.S.A) high speed mixer was used to homogenize the oil–water emulsion. The mixer was set at 2.5 speed (approximately 14,500 rpm) and oil was added into the beaker in small portions (3 mL oil/ 10 s). The auto–ranging ohmmeter (Craftsman Model 82334) was used to detect the transition time when the emulsion changes from oil–in–water to water–in–oil type (i.e. from readable Ohm value to zero value). The total weight of oil was then recorded and used to calculate the emulsion capacity.

$$\text{Emulsion capacity, g oil/g protein} = \frac{\text{Weight of oil}}{\text{Weight of protein}} \text{x} 100$$

2.2.9. Gelling capacity

Gelling capacity test followed the reported method of Bilstein et al. (2008). The gel forming properties of the sample were determined by mixing 2 g of sample with 10 mL of distilled water in 50 mL graduated plastic test tube using ULTRA–TURAX T25 BASIC S1 (IKA–WERKE, U.S.A). The solution was then stored overnight at 4^{0}C. After storing, the solution was heated to a core temperature of 72^{0}C and again was stored overnight at 4^{0}C. The gel creation capacity was evaluated by determination of the remaining free clear water both at normal condition and centrifuge condition, i.e. 3,000 rpm (7,500 x g) for 5 mins using Beckman J2 HS centrifuge (Beckman Coulter, U.S.A). The gel is formed if at normal condition, the content inside the test tube does not slip out of the tube in upside down position.

2.2.10. Rapid viscosity analysis (RVA)

Rapid viscosity analysis is usually used to measure viscosity of starch and to obtain information about starch gelling, i.e. pasting temperature, peak viscosity, and gelling behaviors. This method is not sensitive to small changes in viscosity, i.e. the resolution is poor, but is very useful in terms of providing information about sample behavior to temperature and shear force. Also, it can provide dynamic measurements of sample's viscosity over a period of time. Therefore, it was used to study the pea protein's behavior in this project.

The pea samples were prepared in 10% solution with distilled water. This solution (25 mL) was then transferred to RVA aluminum container. This container was placed into the equipment with plastic propeller attached. Then the equipment was run using following settings:

Table 6. The RVA profile for pea protein viscosity* analysis.

Step	1	2	3	4	5
	Maintain	Heat	Maintain	Cool	Maintain
Temperature, ^{0}C	$25 \rightarrow 25$	$25 \rightarrow 90$	$90 \rightarrow 90$	$90 \rightarrow 25$	$25 \rightarrow 25$
Time, mins	5	3	5	4	5
Propeller speed, rpm	200	200	200	200	200

* Viscosity values were recorded at 3 second intervals as a function of time.

2.2.11. Cake baking method

Cake formulas were self–developed to adopt the use of different sample, i.e. PPI, TGase–treated PPI, and egg. Based on the preliminary research, the final cake formulas were decided as tabulated in Table 7.

Table 7. Cake formulas for one 20 cm diameter cake.

Ingredients at 21 ± 1^{0}C	Weight, g		
	Egg Formula	PPI Formula	TGase–treated PPI formula
Cake flour	130	130	130
Baking powder	7	7	7
Shortening	50	50	50
pea protein	–	6 g PPI	6 g TGase PPI
Water	45	115	115
Fresh egg (whole)	69	–	–
Sugar	100	100	100
Salt	3	3	3
Vanilla powder	3	3	3

Cake baking procedure was adopted from a cookbook. We replaced egg component by an appropriate amount of PPI and TGase–treated PPI which had at least 80% protein. This made an equal formula compared to egg formula in terms of protein content.

For cakes with fresh egg, water was added to egg and whipped until uniform using a hand mixer. Then, sugar, salt and vanilla powder were added and mixed for 1 minute at medium speed, following by the addition of shortening, cake flour and baking powder. The mixture was further mixed for an additional 2 mins before being transferred into pre–sprayed pan. Finally, sample was baked at 350^{0}F for 25 mins in a preheated oven, cooled to room temperature (approximately 1 h) and cake heights and other parameters measured.

For cakes with PPI and TGase–treated PPI, 6 g of isolates was used. The pea isolates was first dissolved in water and mixed for 1 minute using hand mixer at medium

speed. Then, sugar, salt and vanilla powder were added and mixed for 1 minute at medium speed, following by the addition of shortening, cake flour and baking powder. The mixture was further mixed for an additional 2 mins before being transferred into pre–sprayed pan. Finally, sample was baked at 350^0F for 25 mins in preheated oven, cooled to room temperature (approximately 1 h) and cake heights and other parameters measured.

2.2.12. Cake measurement

In order to obtain cake symmetry, and uniformity of cakes, samples produced from above baking method were used as they have 20–cm diameter. The method used in this determination was the layer cake measuring template method (Approved Method 10–91 AACCI 2000). Generally, the cake was cut vertically through the center and then placed with cut surface down on the template, center, and aligned with baseline of template. Height of cake at different positions was read to nearest 0.1 cm (Figure 7).

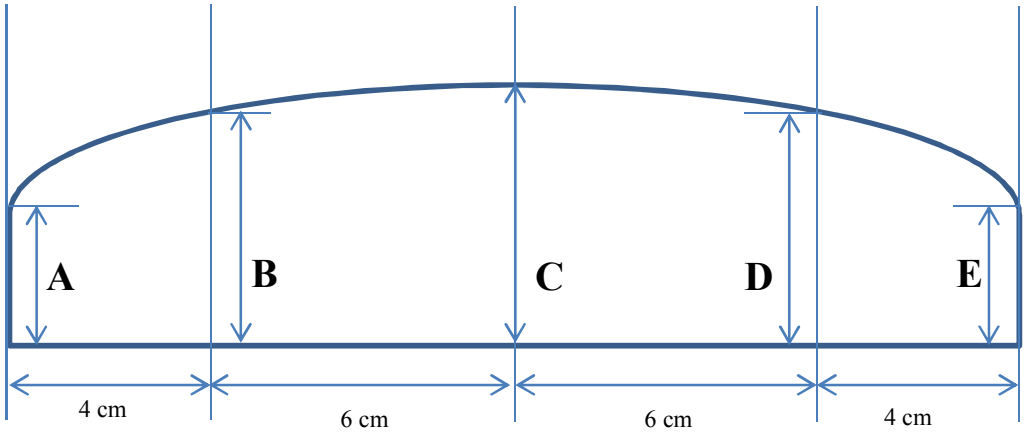


Figure 7. Positions where cake heights were measured.

2.2.13. Texture analysis

Cake texture was analyzed using a Brookfield LFRA texture analyzer with procedure provided by Brookfield with little modification. Cake was first cut into cubes

of 26 mm x 26 mm x 35 mm from the base of the cake (the original dimension was 26 mm x 26 mm x 45 mm but due to the fact that some of our sample has height smaller than 45 mm, we decided to follow the mentioned dimension). This was to standardize all samples. In cake texture analyzing, the LFRA was set to total profile analysis (TPA) mode; trigger load was set to 5 g; test speed was chosen at 1 mm/s; and load cell was 4.5 kg. The plunger used for cake testing was 50.8 mm Φ Perspex cylinder. Samples were test for deformation with target value of 50% in two cycles. Hardness, springiness and adhesiveness were three parameters measured in the test.

2.2.14. Cookie formula and bake quality

Cookie formula in this project was developed based on the formula provided in a cookbook. Briefly, ingredients were weighed in amounts indicated in Table 8. Butter, sugars, and salt were first mixed together in mixing bowl using hand mixer until uniform. Egg/pea slurry was then added and mixed for 1 minute. Finally, all–purpose flour and baking soda were added and mixed for an additional 2 minute until batter was uniform. Batter (12 g) was scooped to make cookies. Cookies were placed in a 375^0F oven for 7 mins. After baking, cookies were removed from the oven, cooled for 5 mins and removed from baking sheet.

After 30 mins, cookies quality was tested following guide lines in Approved Method 10–53.01 AACCI. Eight cookies were laid edge to edge and width measured. Then re–measurement was taken after a quarter turn with three repeats. Cookies were finally stacked in different order to measure height.

Table 8. Cookie formulas for preparation of 35 cookies.

Ingredients at 21 ± 1^{0}C	Weight, g		
	Egg formula	PPI formula	TGase–treated PPI formula
All–purpose flour	175	175	175
Butter	113	113	113
Sugar	75	75	75
Brown sugar	56	56	56
Fresh egg (whole)	23	–	–
Pea protein	–	3 g PPI	3 g TGase PPI
Water	–	22	22
Salt	1.5	1.5	1.5
Baking soda	2.7	2.7	2.7

2.3. Sensory evaluation

The sensory evaluation for consumer acceptability was chosen. Both cake and cookies samples were evaluated. Samples with or without pea protein isolates as an egg replacer samples were tested by a sensory panel of at least 50 panelists (following IRB protocol # AG11039). Nine–point hedonic rating scale was used to determine acceptability from "like extremely" to "dislike extremely". This sensory test measured the consumer acceptability to our test samples where 9 was like extremely and 1 was dislike extremely (Figure 8). Briefly, sequential monadic test was the method chosen to present samples to panelists, meaning all samples were presented in sequence and to be assessed one at a time (Carpenter et al. 2000). Each sample was labeled randomly with a three digit code and each set of samples, consisting of three samples each in duplicate, i.e. six samples per set, was presented in random sequence, and one at a time. Each panelist evaluated one set of samples and scores on product attributes, i.e. appearance, texture, flavor, and overall acceptability, based on the hedonic scale (Figure 8). Data

from this test was analyzed using ANOVA software to provide consumer acceptability on tested samples.

Sensory Evaluation of Pea Fortified Cookies/Cakes

SAMPLE NUMBER: _____

Please evaluate the cookie samples for the following qualities: Flavor, Texture, Appearance and Overall Acceptability (i.e. liking). Make an X on the appropriate line. Please give comments in the space provided below each quality if desired.

APPEARANCE:
——————— like extremely
——————— like very much
——————— like moderately
——————— like slightly
——————— neither like nor dislike
——————— dislike slightly
———————dislike moderately
———————dislike very much
———————dislike extremely

COMMENTS:

FLAVOR:
——————— like extremely
——————— like very much
——————— like moderately
——————— like slightly
——————— neither like nor dislike
——————— dislike slightly
——————— dislike moderately
——————— dislike very much
——————— dislike extremely

COMMENTS:

TEXTURE:

——————— like extremely
——————— like very much
——————— like moderately
——————— like slightly
——————— neither like nor dislike
——————— dislike slightly
———————dislike moderately
———————dislike very much
———————dislike extremely
COMMENTS:

OVERALL ACCEPTABILITY:

——————— like extremely
——————— like very much
——————— like moderately
——————— like slightly
——————— neither like nor dislike
——————— dislike slightly
——————— dislike moderately
——————— dislike very much
——————— dislike extremely
COMMENTS:

Figure 8. Score sheet used in sensory evaluation.

2.4. Experimental design and statistical evaluation of the data

The experimental design used in this project was a randomized complete block design (RCBD) for optimum extraction pH determination, precipitating pH determination, flour–to–water ratio optimization, extraction time optimization, TGase treatment and acceptance study involving cakes and cookies. Each treatment appeared one time in a block and was completed three times except cake and cookie acceptance studies in which treatments were repeated twice in one block, which refers to one panelist. All data was analyzed by analysis of variance (ANOVA) and mean significant difference was tested by least significant differences (LSD) method using SAS program. A confidence level of 5% ($P<0.05$) was used to establish significant differences among the means.

Functional properties evaluation was conducted three times and presented as mean ± standard variation (SD) and confidence intervals were calculated at 95%.

The protein isolates showing the best functional characteristics were carried out in duplicate in cake and cookie systems. The data was statistically evaluated using ANOVA and LSD at the confidence level of 5%.

RESULTS AND DISCUSSION

1. EXTRACTION PROCESS OPTIMIZATION

1.1. Yellow pea flour characterization

Protein content, moisture content, starch content, ash level, degree of foreign matter/contaminants, fat content, seed's size, and endosperm color are yellow pea quality parameters. However, protein quality is the most important since protein isolation was the target of this research. Split yellow pea flour contained 25.8 ± 0.24% (dry basis) protein. This value was in agreement with literature values reported to be in the range of 20–35% (El–Adawy et al. 2003; Schatz and Endres 2009; Boye et al. 2010). Other tested factors are summarized in Table 9. Although not measured, starch, fiber, and lipid make up the remaining pea flour composition.

Table 9. Yellow pea characterization.

Parameters	Value,% (db)*
Protein content	25.8 ± 0.24
Moisture content	11.1 ± 0.07
Ash content	2.5 ± 0.03

* Each value is an average of three determinations ± SD.

Flour size distribution or particle size plays an important role in food manufacturing. Size distribution implies size uniformity of flour particles and it is important because of its great impact on flour hydration, water distribution, and eventually the quality of the final product. The milling process used to produce the flour resulted in particles that were predominantly in the 177 μm to < 149 μm levels (Table 10).

Table 10. Yellow pea flour size distribution.

Sieve no.	Size, mm	Average, %
20	0.841	0.9 ± 0.14
80	0.177	38.9 ± 1.48
100	0.149	12.5 ± 2.04
<100	< 0.149	47.8 ± 1.95

There was no standard definition for the size of individual flour particles found in literature, thus it was necessary to propose a size classification based on current practices. Particles left on sieve no. 20 (0.841 mm) were considered extra–large particles; particles passing through sieve no. 20 but retaining on sieve no. 80 (0.177 mm) were considered large particles; passing sieve no. 80 and retaining on sieve no. 100 (0.149 mm) were classified as medium size particles; and particles that pass through sieve no. 100 (<0.149 mm) were fine particles. According to above particle size classification, a recommended particle distribution for the flour included having no more than 1% of extra–large particle and no more than 50% of fine particles.

Medium particles are proposed to give a good extraction with the least amount of starch contamination. Extra–large particles reduce the interfacial surface thus being proposed to prevent protein extraction. Meanwhile, fine particles have higher interfacial surface which may lead to higher extraction yields. Parthenolide extraction yield was found to be 5 times higher with feverfew particle sizes smaller than 500 μm than that with particle size greater than 500 μm (Fonseca et al. 2006). Coats and Wingard (1950) studied the effects of particle size on oil extraction rate on different samples including soybean, cottonseed, flaxseed, and peanut. The results suggested that larger particle sizes resulted in not only less oil volume extracted but also longer extraction times. In soy

protein isolate extraction, protein recovery can be increased by >30% by decreasing the average particle size of the starting raw material (i.e. defatted soy flour) (Russin et al. 2007). No effect of particle size on yellow pea protein isolate extraction was found but the above studies indicated that particle size reduction could improve the yield. However, finer particles size may be associated with higher starch damage and therefore, resulting in a higher amount of carbohydrate contamination in the final pea protein products. Chau et al. (2007) suggested that particle size had a great effect on a carbohydrate extraction yield from mushroom where 10 μm or smaller particle size gave 10 times higher yield compared to particle size of about 500 μm. A reason for the increase in carbohydrate yield may be due to the amount of starch damage. Di Stasio et al. (2007) concluded that starch damage is a consequence of the physical effects taking place during milling. Thus, the more energy inputs, i.e. required to obtain smaller particle sizes, the more severe physical effects exerted on the pea seed, resulting in more starch damage. Therefore, neither too many extra–large particles nor too many fine particles were desired. Pea flour in this project was obtained from a Fitz mill, which is a high–speed screen hammer mill with flat hammers for impact (Snow et al. 1997). Hammer mill is commonly used in the food industry to grind non–wheat material including fibrous and high oil containing materials. However, due to the simplicity of its internal classifier (the screen), the Fitz mill cannot efficiently control the particle size distribution of its output as can roller mills. This was the reason why pea flour in this project had a high level of fine particles. No attempt was made to separate particles prior to extraction as this might limit protein recovery by eliminating a fraction.

1.2. Extraction pH

Protein solubility is affected by many factors. These factors are classified into two groups: factors belonging to protein's characteristics such as amino acid composition, protein structure, i.e. denatured or native, and factors belonging to the medium such as pH, saline type and concentration, temperature, pressure, and protein concentration (Machado et al. 2007). Of all extraction medium factors, pH and saline concentration are the two most common factors utilized in the protein extraction process. This project utilized the former factor to improve pea protein extraction. In general proteins are more soluble in low pH (acid) or high pH (alkaline) values, due to excess of charges of the same signal, producing repulsion among the molecules, and, consequently, contributing to their higher solubility (Pelegrine and Gasparetto 2004). Protein solubility is higher at alkaline pH due to the fact that the number of negatively charged ions at pH values greater than the pI is larger than the number of positively charged ions at pH values smaller than the pI (Fennema 1997). Therefore, alkaline pH was chosen as the extracting pH. Initially, five pH levels had been chosen to evaluate the pea protein solubility, which ranged from 7.0 to 11.0 with 1.0 unit increment. However, the slurry became too thick when the pH went above 10 making it difficult to stir and continue pH adjustment. Therefore, pH levels were narrowed to 10.0 at maximum. Other treatment conditions, i.e. flour–to–water ratio of 1:6, extraction time of 30 mins, and precipitating pH of 4.5 were held constant as a means to evaluate pH. Adjustment of the pH to resolubilize the precipitated protein was skipped during this phase of the evaluation.

Increasing extracting pH resulted in an increased protein recovery rate, or protein yield in other words (Table 11). This was reasonable as pH moves towards extreme

alkaline or acid regions, the protein solubility increases, resulting in more protein present in aqueous phase and eventually, more protein in final extracts. Protein recovery rate was found to be least at pH 7.0 and highest at pH 10.0. From this trend, it can be implied that further increase in pH levels, e.g. pH 11.0 or higher, could result in even higher protein yield. However, it was reported that the increase in viscosity of high pH slurry of rice flour may be attributed to dissolved proteins and non–cellulosic polysaccharides (Lumdubwong and Seib 2000). Therefore, raising pH level could negatively result in lower protein percentage in the final extract due to more soluble carbohydrate contamination and slurry thickness. From pH 8.0 upward, the protein concentration in final extract decreased slightly from 81.1% at pH 8.0 to 79.9% at pH 10.0. This was because increasing pH not only increased protein solubility but also starch swelling and solubility. The more starch contamination in final extracts results in lower protein levels. Alam and Hasnain (2009) evaluated the effect of pH on swelling and solubility of modified starch from taro and found that at pH 2.0 and 10.0 all studied starches had higher solubility and swelling compared to other pH values tested. The pea protein extract data indicated contamination material and therefore, indirectly agrees with literature. Since all extracts had protein levels closed to 80% or higher, they can be classified as pea protein isolates (PPI).

Table 11. Pea protein yields and recovery rates at different extraction pH values.

pH level	PPI/RM* % (w/w, db)	% protein in PPI (w/w, db)	Protein recovery rate** % (w/w, db)
7.0	16.2 ± 0.23	80.9 ± 1.10	55.3 ± 1.01[a]
8.0	17.2 ± 0.24	81.1 ± 0.51	59.3 ± 0.57[b]
9.0	18.2 ± 0.43	80.5 ± 0.59	61.9 ± 1.27[c]
10.0	19.3 ± 0.25	79.9 ± 0.45	65.2 ± 0.98[d]

* PPI: pea protein isolate, RM: raw material.
** Each value is an average of four determinations ± SD. Samples with different letters indicate significant differences ($p < 0.05$).

Although a statistical difference was found in protein yield at different pH levels, PPI functionalities did not change much except for PPI obtained from pH 7.0 extraction (Table 12). Native pH of pea flour was found to be slightly acidic, i.e. from pH 6.35 to pH 6.55, thus the pH 7.0 extraction was the mildest treatment among all, resulting in least protein denaturation.

Table 12. PPI foaming and emulsion properties at different extraction pH values.

pH level	Foaming capacity (%)	Foaming stability (%)	Emulsion capacity (g oil/g PPI*)	Emulsion stability (%)
10	4.5 ± 0.0	11.1 ± 0.0	268.5 ± 7.64	45.8 ± 1.0
9	3.5 ± 1.0	8.5 ± 7.5	260.7 ± 7.24	46.4 ± 0.0
8	3.7 ± 1.5	3.7 ± 6.4	255.1 ± 3.42	47.6 ± 2.1
7	4.5 ± 2.2	14.4 ± 12.9	298.3 ± 16.26	47.0 ± 1.0
LSD	3.2	18.5	21.2	2.0

* PPI: pea protein isolate.

Protein denaturation is possible with any treatment that cleaves hydrogen bonds, and disrupts ionic and hydrophobic interactions (Belitz et al. 2009). When denaturation occurs, a protein unfolds, resulting in increased hydrophobic interactions which

ultimately cause a reduced functionality. As the result, PPIs extracted at alkaline pHs, i.e. 8, 9, and 10, had reduced functionalities compared to that of PPI extracted at pH 7. Alternatively, further increasing in extracting pH towards alkaline side induces a better solubility of the unfolded protein, resulting in a flexible polymer thus increases its functionality (Fennema 1997). This could be used to explain a better functionalities of the PPI extracted at pH 10.0 compared to those extracted at pH 8 and 9. Once pH was shifted towards basic side, foaming capacity, foaming stability, and emulsion capacity increased but emulsion stability decreased (Table 12). Moreover, changing in extracting pH may also alter the protein composition in PPIs, i.e. higher extracting pH induced high molecular weight proteins' solubility and eventually their occurrence in PPIs. Lowering or rasing the pH tends to increase the net charge of proteins towards their maximum (Belitz et al. 2009), thus increasing their solubility. Consequently, PPIs obtain from high pH extraction may have more globulin typed proteins compared to those obtained from lower pH extraction. In contrast, at neutral pH extraction, i.e. pH 7.0, PPIs may contain more albumin typed proteins. This is the second reason causing the change in PPI functionalities.

Standard deviations (SDs) were found to be very high in foaming stability test, indicating the limitation of current method. Currently, there is no approved standard method for protein foaming capacity and stability tests, thus different authors suggested different method for testing protein foaming capacity and foaming stability (Coffman and Garcia 1977; Akintayo et al. 1999; Guerrero et al. 2002; Bildstein et al. 2008). These methods commonly use a slurry of protein that is mixed in a blender and foaming volume recorded. However, each method uses a different protein concentration, mixing speed,

mixing time and the way to handle the foam after mixing. The current method used 0.5% (w/v) protein solution, which is much lower compared to other methods, e.g. Bildstein et al. (2008) used 6% (w/v) protein slurry and Akintayo et al. (1999) used protein slurry ranging from 2% to 10% (w/v). This might affect the foam testing results. Combining all results, PPI extracted at pH 10.0 had the closest overall functionalities compared to PPI extracted at pH 7.0 but had the highest protein yield, therefore, pH 10.0 was considered an optimized extracting pH for yellow pea.

1.3. Precipitating pH (pI)

The protein extracts were prepared from a flour–to–water ratio of 1:6, extracting pH 10.0, and extraction time of 30 mins at room temperature. Precipitating pH was the only factor evaluated. Initially, precipitating pH or isoelectric point (pI) optimizing process had been conducted on pH values ranging from pH 3.7 to 5.0. The preliminary research revealed a narrower range for pea protein precipitation. Thus, smaller increment, i.e. 0.1 pH unit, was used (Table 13). Absorbance (Abs) at 280 nm indicated the amount of protein remaining in supernatant after protein precipitating step. High Abs value indicates high amount of protein present in the supernatant. Thus, the Abs value of supernatant indirectly implies the efficiency of precipitating process. Lower Abs values indicate greater precipitation, i.e. less protein in supernatant, and indicate the pI of the yellow pea protein.

No significant differences were found between Abs at pH values from 4.0 to 4.3 whereas Abs at pH 4.4 and pH 4.5 were statistically different from that at pH 4.3. This indicates that the pea protein pI falls into an even narrower pH range between pH 4.0 to pH 4.3.

Table 13. Absorbance of pea protein supernatant at different pH values.

pH	Absorbance* of supernatant at 280 nm
4.0	0.283 ± 0.005^{ab}
4.1	0.282 ± 0.003^{ab}
4.2	0.280 ± 0.003^{ab}
4.3	0.276 ± 0.006^{a}
4.4	0.288 ± 0.004^{b}
4.5	0.335 ± 0.008^{c}

* Samples with different letters indicate significant differences ($p < 0.05$).

Abs at pH 4.3 had the lowest abs value among all abs in the pH 4.0 to pH 4.3 range, indicating that the pea protein isoelectric point is at pH 4.3 (Figure 9). This value is slightly lower than literature value of legume protein, which is usually at pH 4.5 for soybean, Mediterranean legumes (Pastor–Cavada et al. 2010), butter bean (Guerrero et al. 2002), and pigeon pea (Akintayo 1999). Suat (2005) and Karaca et al. (2011) reported pea protein pI of 4.5. So, it is evident that pH 4.5 is not an empirical value since different species have different protein content, different amino acid and protein composition, and eventually should have different pI. The current experiment focused only on a particular species, which was yellow pea, therefore, the pI was more applicable to the yellow pea compared to a general theoretical pI of 4.5.

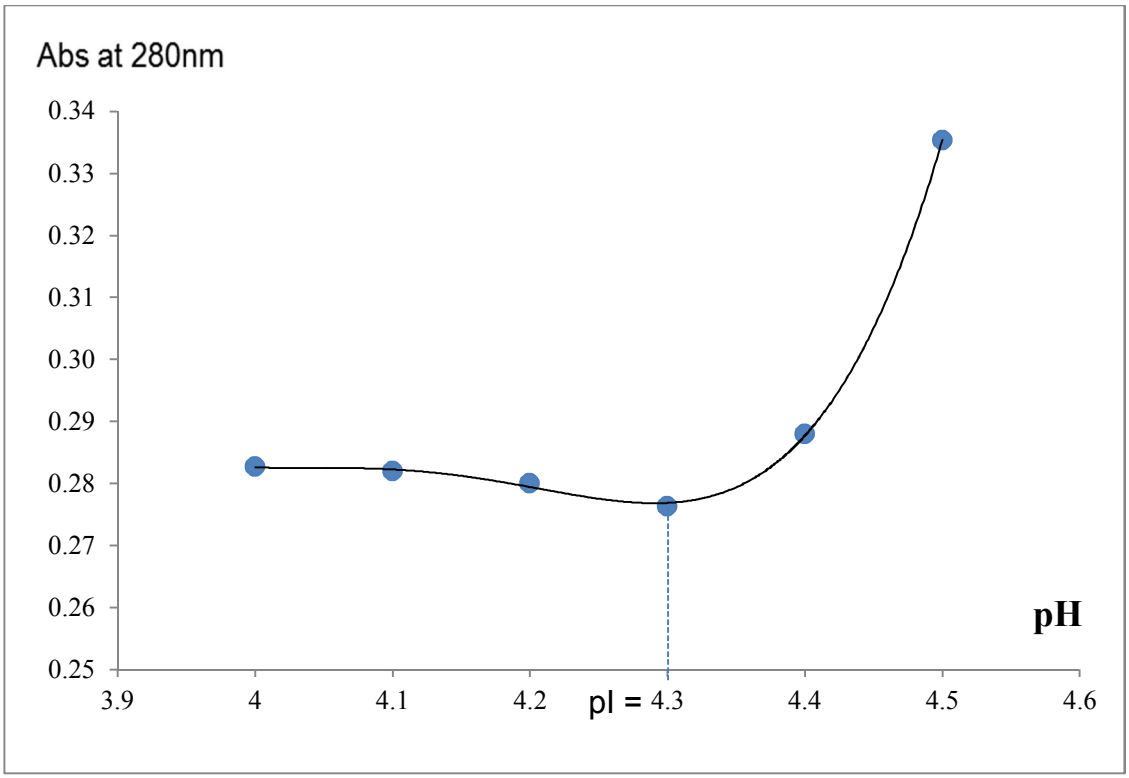


Figure 9. Absorbance of pea supernatant as a function of pH.

1.4. Flour–to–water ratio

Flour–to–water ratio was studied based on the extracting pH and precipitating pH (pI) values determined in previous optimization processes. Extraction time was maintained at 30 mins. The practical ratio normally used for legume protein extraction ranges from "1 to 5" up to "1 to 10" (Rosenthal et al. 1998; Tian et al. 1999; Bildstein et al. 2008; Karaca et al. 2011). In this study, however, the range was extended from a little lower, i.e. "1 to 4", to a bit higher, i.e. "1 to 12", to further investigate if there was any difference in extraction values.

Six different flour–to–water ratios were studied and most of them were not statistically different from each other (Table 14). Only the1:12 ratio produced pea protein yield significantly lower compared to the other treatments. Although no significant

difference was found among the 1:4 to 1:9 ratios, increasing flour–to–water ratio, i.e. more water, resulted in a slight increase in protein yield. However, when reaching 1:10 and higher, increase in flour–to–water led to the reduction in protein yield.

Table 14. Pea protein yield at different flour–to–water ratios.

Flour–to–water ratio	Protein yield* (g protein/20g flour, db)	CV** (%)
1:4	2.89 ± 0.01^{b}	0.25
1:6	2.94 ± 0.05^{b}	1.69
1:8	2.98 ± 0.03^{b}	0.92
1:9	3.09 ± 0.00^{b}	0.03
1:10	3.00 ± 0.03^{b}	0.82
1:12	2.58 ± 0.34^{a}	13.03

* Samples with different letters indicated significant differences ($p < 0.05$).
**CV: Coefficient of Variation.

Flour–to–water ratio is one of the most important factors affecting extraction yield (Anderson et al. 1960). The driving force related to the gradient of the component concentration between the solid and the liquid phases is the main factor governing protein dissociation and/or diffusion (Rosenthal et al. 1998). At low values, increasing flour–to–water ratio creates larger differences in concentration gradient between solid and liquid phases, driving more protein to dissociate from flour and diffuse into the liquid phase, and eventually resulting in an increase in protein yield. This trend was found when flour–to–water ratio was smaller than 1:9. However, at higher values, the gradient concentration between two phases at a particular ratio was still higher but the value differences between ratios were dramatically reduced due to the limitation in original soluble protein amount in the solid phase. Moreover, the significantly high CV value at flour–to–water ratio of 1:12, i.e. 13.03%, indicated the instability of the extraction

process at such a high flour–to–water ratio which may relate to handling of excess water. These two factors combining together may result in the lower protein yield at higher flour–to–water ratio as observed in the flour–to–water higher than 1:10.

The increase in the flour–to–water ratio resulting in reduced protein extraction in wheat flour (Anderson et al. 1960) and soybean flour (Rosenthal et al. 1998) has also been published. In general, our result showed the same trend as literature findings with only slight difference at low flour–to– water ratios. It was also clear that flour–to– water ratios lower than 1:10 gave better protein yield compared to higher water ratios (Table 14).

Flour–to–water ratio of 1:6 was chosen as an optimized flour–to–water ratio since no significant difference in protein yield was observed as water level increased up to 10. Although higher ratios showed slightly better yields, e.g. 1:9 and 1:10 ratios, these yields were not significantly different from that at 1:6 ratio. At lab scale, less equipment capacity, i.e. centrifuge, freeze dryer, shorter time variation, and less loss during extraction were advantages of 1:6 ratio over higher ratios, supporting the choice of 1:6 ratio. However, at industrial scale, it would make sense to go with 1:9 ratio as this ratio gave the highest yield, resulting in the highest profit to producers. But careful consideration should be made in this situation as increasing flour–to–water ratio leads to more requirements in equipment capacity, energy consumption, storage facility and waste water treatment. At 1:4 ratio, result also showed the same protein yield but at this level, the pea slurry was thick and viscous, causing problem in pH adjustment and normal handling.

1.5. Extraction time

Extraction time optimization was carried out under optimized conditions of extracting pH (pH 10), precipitating pH (pH 4.3), and flour–to–water ratio (1:6) as previously discussed. Extraction time did not significantly affect protein yield (Table 15). However, the 30–minute extraction time gave the highest protein yield whereas the other two treatments had slightly lower yields.

Table 15. Pea protein yield at different extraction times.

Extraction time (minute)	Protein yield** (g protein/20g flour, db)	CV* (%)
15	2.85 ± 0.02^{a}	0.69
30	2.92 ± 0.03^{a}	1.14
45	2.87 ± 0.03^{a}	0.95

* CV: Coefficient of Variation.
** Samples with different letters indicate significant differences ($p < 0.05$).

In this study, extraction time did not have a great impact on protein yield. This is in agreement with literature findings where extraction time did not greatly influence the extraction yield. Kongo–Dia–Moukala and Zhang (2011) found that increases of extraction time and extracting pH both resulted in a higher protein extraction but pH and flour–to–water ratio were the two factors that influenced the protein extraction process from defatted corn flour most whereas extraction time had a lesser effect. Abu–Tarboush (1995) suggested that the extraction time was not important to optimum protein extraction if extraction solvent contained sufficient ions for maximum nitrogen solubility. The author found that most of the extractable proteins in karkade flour were solubilized during the first 10 mins. Rosenthal et al. (2008) proposed the same finding where most of the extraction takes place within 5 or 10 mins in protein and oil extractions from soybean.

The oil and protein yields started to reach their constant levels at 13.25 mins of extraction (Rosenthal et al. 2008). Kongo–Dia–Moukala and Zhang (2011) suggested 33–minute extraction time as an optimum value for protein extraction from defatted corn flour. Extraction times longer than the proposed values, therefore, become unnecessary. One possible explanation for the minor impact of extraction time on protein yield was that at high extracting pH, the overall charge on the protein surface was maximized that allows the protein to more readily dissociate and to diffuse into the liquid phase. Therefore, the extraction time became a less important contributor to protein yield. The increase in protein yield with increased extracting pH during this study supported this hypothesis. Since the extracting pH in this section had been optimized at a high level, i.e. pH 10.0, the protein yield became less dependent on the extraction time within the studied range, i.e. from 15 mins to 45 mins.

In general, it can be implied that any extraction time between 15 mins to 45 mins works on pea protein extraction when extracting pH is set at an optimum level. However, between different extracting times, the 30 minute extraction was chosen as an optimized extraction time. It was chosen with the consideration of our current equipment condition as well as a slightly higher yield at 30 minute extraction compared to that at 15 and 45 mins.

Four variables that have the greatest impact on protein yield had been studied and evaluated. Optimum values are presented in Table 16.

Table 16. Operating parameters for optimized extraction process.

Parameter	Settings
Extracting pH	10.0
Precipitating pH	4.3
Flour–to–water ratio	1:6
Extraction time	30 mins

The hypothesis used in this optimization process was that four variables are independent and their impact on the extraction process does not interfere with each other. In fact, results obtained from these experiments supported this hypothesis. However, the proposed optimum extraction process had several limitations. Setting extraction time to 30 mins did not affect the protein yield as no statistical difference was detected between 15–minute– and 30–minute extractions. However, 30 minute extraction consumed more energy, i.e. energy to operate stirrer, and reduced process capacity compared to 15 minute due to the time extension of the overall process. This is considered the first limitation of the proposed optimum process. This limitation can be corrected with the upgrade of equipment or in real production where every factor will be optimized to maximize profits. One cycle extraction is the last limitation of the reported optimization. The protein content in our extraction process was comparable to the upper values in literature findings where pea protein content ranged from 84.9% (Fernandez–Quintela et al. 1997) to 90.1% (Chakraborty et al. 1979) using the same extraction method but different processing conditions. However, recovery rate would have been improved if multi–step extraction was used. Moreover, the discard of supernatant after isoelectric precipitation in the freeze drying method caused the loss of approximately 6.2% in protein recovery rate according to our research findings.

The optimized extraction process was examined on samples with different storage times, i.e. sample at 48h after milling (zero time) and samples after 1–year–storage (1 year time), to test the effect of storage time on protein extraction process. The recovery rates were found to be 63.4 ± 1.41% and 65.2 ± 0.98% for 1–year–sample and fresh sample, respectively. A slight reduction of 1.8% in recovery rate was found in the aged sample, indicating storage time did have some negative impact on protein yield but this effect was not severe. In contrast, the protein content in PPI obtained from 1–year–old sample was higher than that of fresh sample, i.e. 87.5 ± 0.26% vs. 79.9 ± 0.45%. The higher protein content in 1–year–old sample could be the result of several factors including skill, and chemical and physical changes in flour due to the impact of storage condition. Improvement in hands–on experience built up over the year may account in better yield and purity of the protein extract. The protein yield and purity could be affected by processing conditions according to Russin et al. (2007). As the fresh sample was extracted under non–optimized conditions whereas the 1–year–sample was extracted under optimized process, it makes sense that the latter had better protein purify compare to the former. In contrast, longer storage may associate with oxidation, denaturation, destruction, and modification of components in pea flour including protein. The effect of these activities remained unclear but according to the results, they might have a bigger negative impact on protein yield than on protein purity.

Effectiveness of extraction process was evaluated by SDS PAGE. Typically, polyacrylamide gel electrophoresis (PAGE) is used to determine the size of proteins. Briefly, when proteins are passed through a polymerized acrylamide gel, small proteins will travel more quickly than larger proteins, thus allowing for their separation

(Campbell–Platt 2009). To isolate mass as the only variable among proteins in a sample, sodium dodecyl sulfate (SDS) is used to completely unfold proteins and to normalize charge to mass ratio (Campbell–Platt 2009). In the situation where proteins are multiple unit molecules, the reducing agent β – mercaptoethanol is also added to break disulfide bonds to completely unfold such proteins. This ensures that both compact and bulky proteins migrate through the gel at a rate only proportional to their mass and unaffected by native molecular shape or native charge (Campbell–Platt 2009). A gel without β – mercaptoethanol is called the non–reducing gel whereas a gel with β – mercaptoethanol is called the reducing gel.

The 12% non–reducing gel showed that the extracted PPI had a very similar protein profile compared to that of original pea flour (Figure 10). High molecular weight protein fraction in PPI showed an exact pattern as those in pea flour, suggesting that pH 10.0 was efficient at extracting most of the proteins from peas including high and low molecular weight protein fractions. However, in medium weight fraction, one protein band (~ 45,000Da) was missing in PPI protein profile as circled in Figure 10.

These proteins were neither a part of globulin or albumin type proteins in pea nor subunits of such protein types. The major albumin protein contains two polypeptides with molecular weights of ~25,000 Da whereas the minor albumin protein has a molecular weight of approximately 6,000 Da (Rao et al. 1989). The major globulins found in pulses are legumins (11S) and vicilins (7S). 11S Legumins have hexameric quaternary structures with acidic (molecular weight of ~40,000 Da) and basic (molecular weight of ~20,000 Da) subunits (Boye et al. 2010). The 7S vicilins have a trimeric structure with molecular weights of 175,000–180,000 Da (Boye et al. 2010). It is suspected that denaturation or

polymerization of such proteins may be a cause of their absence in the non–reducing SDS–PAGE gel.

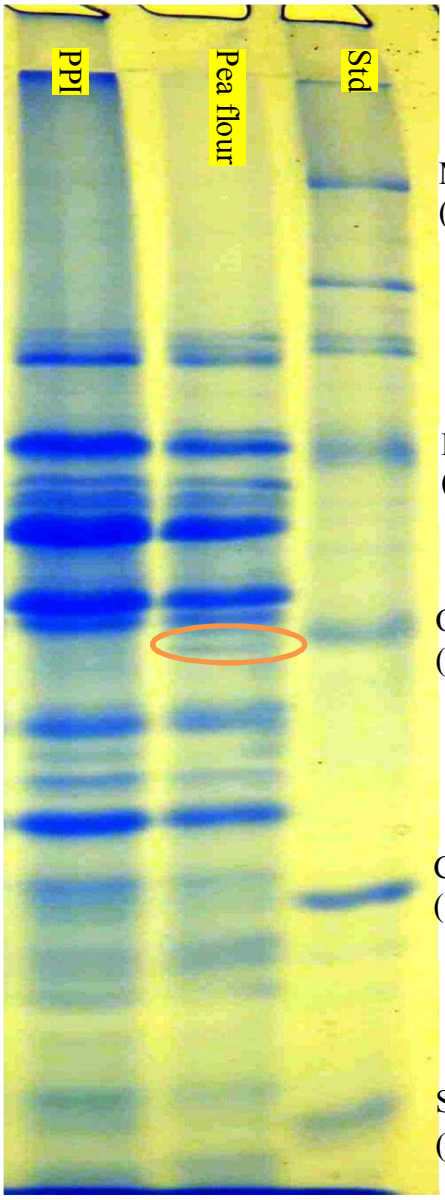


Figure 10. Protein fractions in pea protein isolate compared to those in pea flour separated by 12% non–reducing SDS PAGE.
Std: standard; PPI: Pea protein isolate.

The reducing SDS–PAGE result (Figure 11) reinforced the explanation as both pea flour and PPI protein profiles were identical. The missing band at position 45,000 Da

in the non–reducing gel appeared on the PPI protein profiles in the reducing gel, suggesting that the polymerization or the association with other proteins was the cause of their absence in the non–reducing SDS–PAGE. Under the specific condition of reducing gel, i.e. with the presence of β – mercaptoethanol, multiple–unit protein molecules were completely unfolded and disassociated into single unit molecules (Campbell–Platt 2009), leading to the presence of the previous missing band in the non–reducing gel.

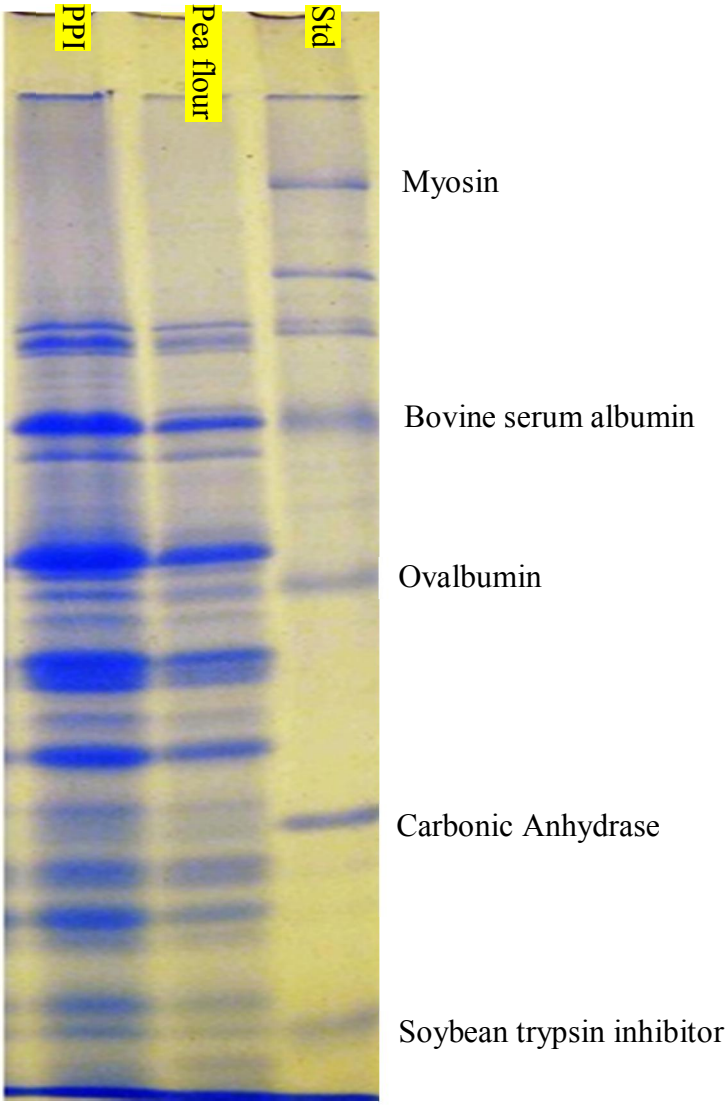


Figure 11. Protein fractions in pea protein isolate compared to those in pea flour separated by 12% reducing SDS PAGE.
Std: standard, PPI: pea protein isolate.

2. TRANSGLUTAMINASE MODIFICATION OF PEA PROTEIN

According to literature, PPI showed poor gelling property compared to other legume and pulse protein isolates (Soral– Smietana et al. 1998; O'Kane et al. 2005; Shand et al. 2007; Bildstein el al. 2008), therefore PPIs were treated with TGase to improve their gelling properties. Since PPIs are very complicated systems containing many different protein fractions at various concentration and different solubility, none of the current enzyme essays is ideal for studying TGase effectiveness in pea protein systems. SDS PAGE can be used to evaluate the effectiveness of TGase in cross–linking proteins but it cannot be used to determine functionality. However, RVA, in combination with SDS PAGE, can provide much more useful information.

The effect of TGase on functionalities of pea protein extracts could be greatly biased if pea protein extracts were contaminated with high amounts of soluble starch. Starch and other food carbohydrate are usually used to stabilize food products such as emulsion, foam, and frozen dairy products (Wang and Cui 2005); thus, the presence of starch in protein extract may lead to an inaccurate judgment about a role of TGase treatment on pea protein extracts' functionality. So, it is necessary to minimize an amount of starch if present in final pea protein extracts. The minimization could be a physical process, e.g. centrifugation to remove soluble starch from protein pellet as used in the freeze drying method, or a biological process, i.e. enzymic degradation as used in spray drying method.

2.1. Soluble starch degradation

The second extraction approach was designed in the way that the second centrifuge was eliminated and the whole supernatant was spray–dried, raising the possibility of starch impact on overall functionality of final TGase–treated pea protein products. It was, therefore, necessary to degrade starches present in the supernatant prior to the drying process to minimize their impact on the final protein product's functionality. α–Amylase and amyloglucosidase were used to break starch into smaller fractions and to degrade smaller fractions to maltose and oligosaccharides, respectively.

Total starch result showed that, after treatment, the amount of starch present in spray–dried samples was similar to that of freeze–dried samples, i.e. 0.15 ± 0.02% vs. 0.15 ± 0.03% for control samples and 0.28 ± 0.10% vs. 0.36 ± 0.04% for TGase–treated samples, proving the current enzyme dosage and reaction conditions efficiently degraded soluble starches. Thus, the impact of soluble starch on final product was limited and the functionality improvement was mainly a result of TGase activity.

2.2. Polymerized protein formation

According to the manufacturer (Ajinomoto), the optimum pH for TGase Activa TI falls between 6.0 and 7.0. The non–reducing SDS–PAGE result agreed with the manufacturer's suggestion (Figure 12). The TGase treatment at pH 6.0 and 7.0 gave a significantly higher amount of polymeric protein, which were retained in the well and could not travel through the stacking gel. TGase treatment at pH 4.3 and pH 5.0, i.e. sample 4.3 and sample 5.0, respectively, did show some polymeric protein but with very limited quantity.

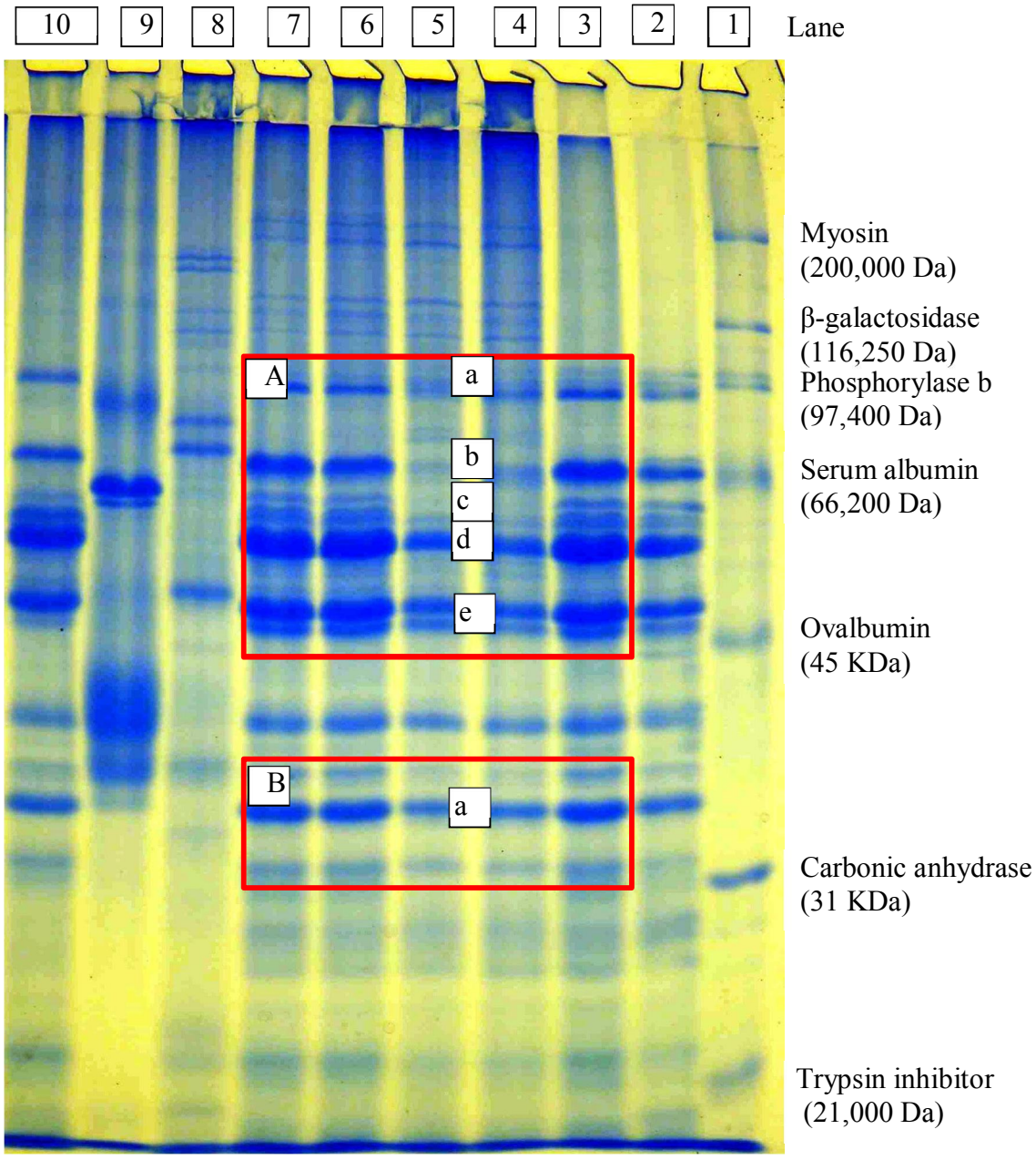


Figure 12. Protein fractions in TGase–treated pea protein isolates separated by 12% non–reducing SDS PAGE.
From right to left: 1. Standard; 2. Pea flour; 3. PPI (control); 4. TGase–treated sample (at pH 7.0); 5. TGase–treated sample (at pH 6.0); 6. TGase–treated sample (at pH 5.0); 7. TGase–treated sample (at pH 4.3); 8. Commercial soy protein isolate; 9. Egg albumin; 10. Commercial PPI (Roquette, Nutralys F85M). Capital letters, i.e. A, B, indicate different molecular weight regions while lower case letters, i.e. a, b, c, d, e, indicate protein band positions.

Of all treatments, sample 4.3 had the least polymeric proteins formation. The SDS–PAGE also suggested that the large molecular weight proteins (~ 90,000 Da) and medium weight proteins (~50,000 – 80,000 Da) were main substrates for TGase catalyzed reaction whereas most of the proteins in the low molecular weight fraction (< 45,000 Da) were not involved in the reaction. Samples with TGase treatment at pH 6.0 and pH 7.0, i.e. sample 6.0 and sample 7.0, respectively, showed the complete disappearance of protein band at ~ 55,000 Da position (position A–c) and the significant reduction in band intensity at position A–a, A–b, A–d, and A–e (Figure 12). In contrast, no intensity reduction or disappearance of any protein band within the "45,000 to 100,000 Da region", i.e. region A, were observed in sample 5.0 and sample 4.3 compared to the control PPI. The only low molecular weight proteins that participated in the TGase reaction were found at position B–a in "31,000 to 35,000 Da region", i.e. region B, of sample 6.0 and sample 7.0 (lanes 4 and 5 in Figure 12), where the intensity of protein bands was reduced compared to those of PPI and samples 5.0 and sample 4.3. Based on the positions, A–a, A–b, A–d, A–e, and B–a were identified as lipoxygenase, convicilin fractions, legumin non–reduced, and vicilin fractions, respectively (Barac et al. 2010). This means that globulin, i.e. legumins, vicilins and convicilins, is the main protein fraction involved in TGase catalyzed interactions. In general, the result is in agreement with literature where albumins and globulins were found to be the main substrates for TGase catalyzed reaction (Macro et al. 2007). Reason for not seeing all albumin bands in the gel is that albumins have very low molecular weights (Croy et al. 1984; Rao et al. 1989), thus require a short time to complete the distance from the well to the bottom of the gel. Since the long running time was required to get a good resolution on high

molecular weight fractions, it is not able to get a good resolution on albumin fraction as all albumins accumulated at the bottom of the gel.

Reducing SDS PAGE did not provide much information about the effect of TGase treatment on protein molecular weight's changes. However, the result revealed that there were disassociations of proteins in the medium weight region, i.e. region between serum albumin (66,200 Da) and Ovalbumin (45,000 Da). Notably, there were a complete absence of protein bands at region A (Figure 13) compared to the non–reducing gel (Figure 12), suggesting that these proteins have polymeric structures in which subunits are linked together by disulfide bonds. According to Barac et al. (2010), this is a position of non–reduced legumin which consists of an acidic subunit of ~40 kDa and a basic subunit of ~20 kDa, linked by a single disulfide bond (Gueguen and Barbot 1988). The increase in intensity of protein bands at region C, ~40 kDa, and the appearance of protein band at position b, ~20 kDa, in reducing gel were evidences of this proposed disassociation (Figure 13).

There were two new protein bands in region B in the PPI, samples 5.0 and 4.3, but not in samples 6.0 and 7.0, suggesting that the original polymeric protein participated in the TGase reaction at pH 6.0 and 7.0 and the new covalent bond formations prevented the disassociation of such a protein under the particular reducing condition used in this experiment. This is reasonable as the reducing agent, i.e. β – mercaptoethanol, in SDS–PAGE is only efficient to reduce intra– and inter–disulfide bonds (Khan and Nygard 2003, Campbell–Platt 2009). Since the bonds formed by TGase were between the carboxyamide groups of peptide–bound glutamine residue and a variety of primary

amines (e.g. protein/peptide–bound lysine) (Ohtsuka et al. 2000), they are resistant to reducing agents such as β – mercaptoethanol.

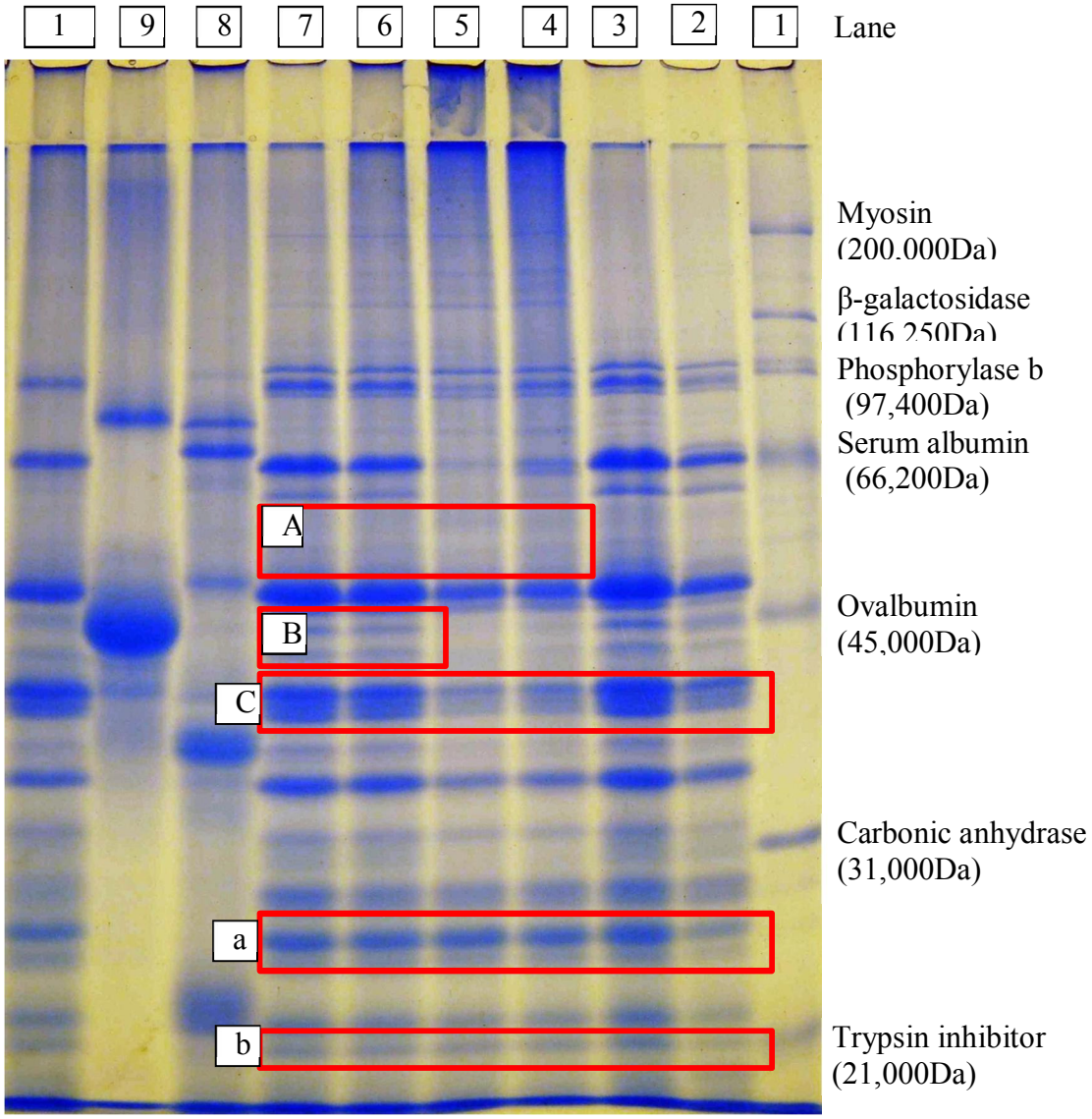


Figure 13. Protein fractions in TGase–treated pea protein isolates separated by 12% reducing SDS PAGE.
From right to left: 1. Standard; 2. Pea flour; 3. PPI (control); 4. TGase–treated sample (at pH 7.0); 5. TGase–treated sample (at pH 6.0); 6. TGase–treated sample (at pH 5.0); 7. TGase–treated sample (at pH 4.3); 8. Commercial soy protein isolate; 9. Egg albumin; 10. Commercial PPI (Roquette, Nutralys F85M). Capital letters, i.e. A, B, C, indicate different molecular weight regions while lower case letters, i.e. a, b, indicate protein band positions.

Between two treatments at pH 7.0 and pH 6.0, the level of polymerized proteins cannot be differentiated if only based on SDS–PAGE result. In other words, the SDS–PAGE result did not tell which treatment was better than the other in terms of linking small protein molecules to bigger molecular weight aggregates.

2.3. Viscosity behaviors

TGase promotes the cross–linking of protein molecules via formations of inter–molecular covalent bonds between Glu and Lys (Motoki and Seguro 1998). The formation of polymeric proteins can increase the viscosity of the pea protein solution, which can be detected by a rapid viscosity analyzer (RVA). The accuracy of current RVA equipment used in the lab is equivalent to ± 150 cP, (Newport Scientific, http://www.newport.com.au/products/brochures/rva_starchmaster2.pdf) whereas the viscosity of pea protein slurries were usually lower than 300 cP. Therefore, the RVA method is only used to detect a trend of protein behavior in the solution.

Fresh and reconstituted TGase–treated protein slurries were the two types of protein slurries evaluated. The fresh protein slurries were protein precipitates collected after the final centrifugation step that were directly re–slurried with distilled water, final pH adjusted to 7.0, total volume adjusted with distilled water, and tested without undergoing the drying step. The reconstituted protein slurries involved diluting the protein residues collected after the final centrifugation with distilled water, final pH adjusting to 7.0, final volume adjusting, drying and rehydrating with distilled water before the RVA test. All protein slurries had the same 10% protein concentration.

RVA result of fresh protein slurries showed that sample 6.0 had the highest viscosity among all samples (Figure 14). It started with low viscosity compared to that of

sample 7.0, but increased when heated, and finally reached its peak during the cooling. Although starting with highest level of viscosity, the viscosity of sample 7.0 decreased after being heated and remained low during the cooling state. Viscosity of samples 5.0, 4.3 and the control did not change throughout the complete RVA cycle. This can be explained based on the formation of polymeric protein aggregates. The gel result showed that only samples 6.0 and 7.0 had a significant amount of polymeric protein aggregates whereas a very limited amount of the aggregates were found in samples 4.3 and 5.0. Consequently, the sample 6.0 and 7.0 had higher viscosities compared to samples 5.0, 4.3 and the control sample. The result was in agreement with literature. Siu et al. (2002) reported that TGase treatment changed flow properties of oat globulin dispersions, indicating increased viscosity and yield stress.

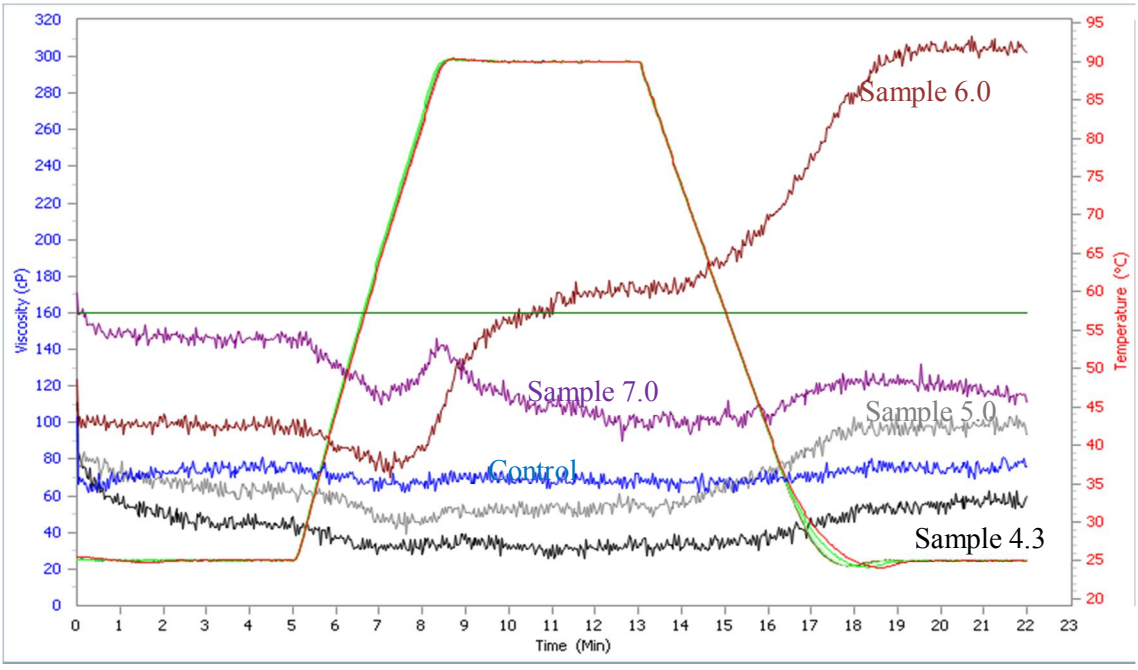


Figure 14. Viscosity profiles for fresh TGase–treated pea protein slurries obtained by RVA.
Control: PPI without TGase treatment; Sample 4.3, sample 5.0, sample 6.0, sample 7.0 are samples with TGase treatment at pH 4.3, 5.0, 6.0, and 7.0, respectively. All the samples and control were prepared from fresh, non–dried pea protein extracts.

RVA results of reconstituted protein slurries showed that sample 7.0 had the highest viscosity among all samples, although sample 6.0 was similar (Figure 15). This observation suggested that the freeze drying process did have some impact on functionality of PPIs. The slow freezing step, which typically associates with the large ice crystal formation, could be the cause for the change in viscosity of sample 7.0. However, the answer to the question of why the slow freezing did not affect the viscosity of sample 6.0 remains unclear.

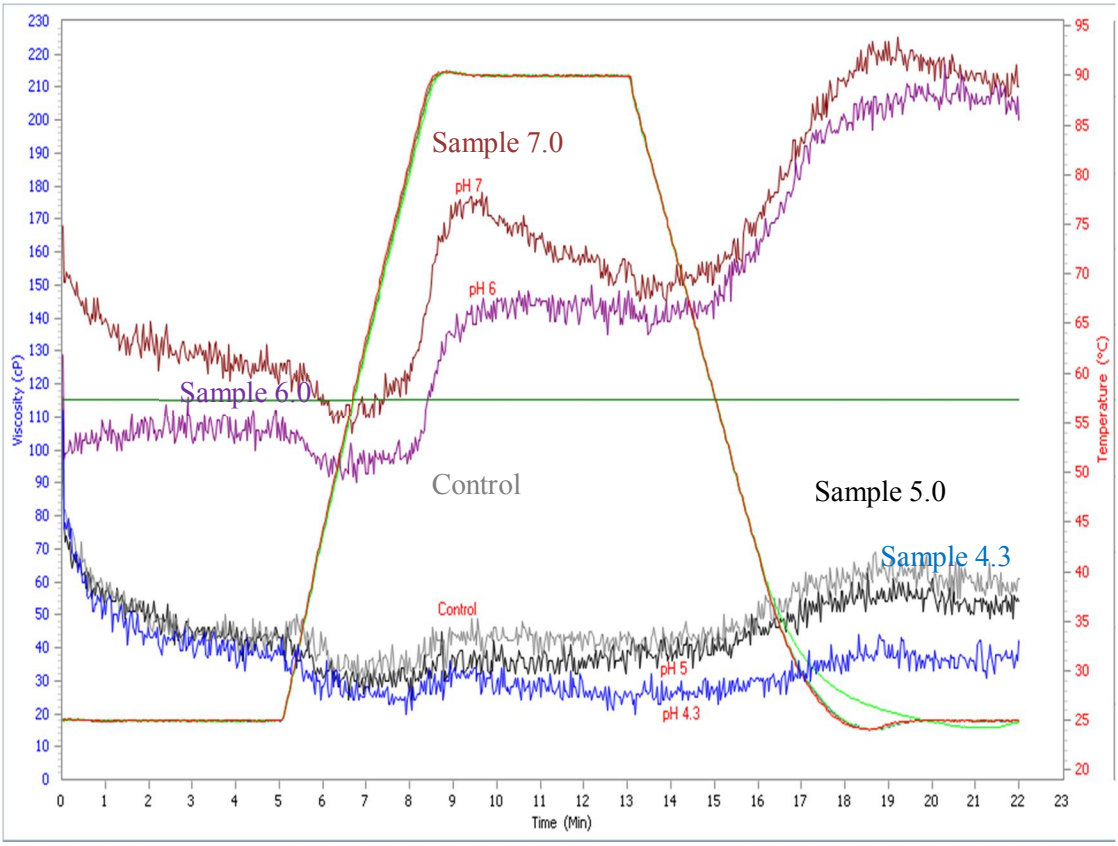


Figure 15. Viscosity profiles for dried TGase–treated pea protein isolate slurries obtained by RVA.
Control: PPI without TGase treatment; Sample 4.3, sample 5.0, sample 6.0, sample 7.0 are samples with TGase treatment at pH 4.3, 5.0, 6.0, and 7.0, respectively.

Although sample 7.0 had the highest viscosity among all samples, its viscosity tended to decrease overtime. On heating, the viscosity of sample 7.0 reached its first peak value after approximately 1 minute at 90^0C but then started to decrease overtime as the temperature remained unchanged (Figure 15). On cooling, it reached its second peak value after approximately 2 mins when temperature reached 25^0C. Meanwhile, the viscosity of sample 6.0 was very stable and was maintained overtime at both heating and cooling steps (Figure 15). This indicated that sample 6.0 performed better than sample 7.0 in viscosity stability while retaining comparable viscosity magnitude.

Again, since TGase did not work well at low pH levels, resulting in very limited protein structure modification, the effect of the freeze dry step on viscosity of sample 5.0 and sample 4.3 was not apparent. The only difference between samples with and without undergoing freeze dry step was the quick reduction in viscosity at the beginning of the measurements of freeze–dried samples compared to fresh samples.

2.4. TGase–treated pea protein functionalities

TGase–treated PPIs were prepared using the first approach, i.e. freeze drying approach, without final pH adjustment. Functionality results of the TGase–treated PPIs at different pH levels suggested that TGase treatment improved functionality of all treated samples in terms of foaming capacity and stability (Table 17). Foaming capacity slightly increased in sample 7.0 and reached max values in samples 5.0 and 6.0. Sample 4.3 had higher foaming values compared to that of the control but considering the SD value, this was not significantly different. Similar finding was found with foaming stability where sample 6.0 had the highest foaming stability, following by sample 5.0. Sample 7.0 and sample 4.3 had similar foaming stability and all were higher than that of the control.

In terms of emulsion capacity and emulsion stability, TGase treatment did improve those functionalities in sample 7.0, sample 6.0 and sample 5.0 but not in sample 4.3. Among all samples, sample 6.0 showed the best emulsion stability but only lower emulsion capacity compared to that of sample 7.0. However, this difference was small, i.e. approximately 3%, and was not significantly different (Table 17).

Table 17. Foaming and emulsion properties of PPIs at different TGase treatments.

pH level	Foaming capacity (%)	Foaming stability (%)	Emulsion capacity (g oil/g PPI*)	Emulsion stability (%)
Sample 7.0	3.0 ± 0.0	11.1 ± 9.6	289.1 ± 14.59	47.0 ± 1.0
Sample 6.0	3.3 ± 0.3	34.9 ± 7.3	280.4 ± 9.78	50.6 ± 4.1
Sample 5.0	3.3 ± 0.6	20.8 ± 11.0	281.4 ± 31.98	48.2 ± 5.4
Sample 4.3	2.5 ± 1.0	11.4 ± 10.3	260.3 ± 4.84	46.4 ± 4.3
Control	2.3 ± 0.3	6.7 ± 11.5	267.3 ± 3.71	47.0 ± 1.0
LSD	1.1	20.8	30.6	4.7

*PPI: pea protein isolate.

The results indicated that TGase did not work well at the pI, i.e. pH 4.3, resulting in a functionality reduction of sample 4.3 compared to other treated samples. At pI, most pea proteins are precipitated out of the solution. The neutral overall surface charge induces hydrophobic interaction between protein molecules causing precipitation thus limiting their ability to participate in the TGase–catalyzed reaction. Moreover, the low pH level of the medium may also denature the TGase and impact the ionization of prototrophic groups in TGase active site which consequently affects both catalytic reaction and binding of the substrates to the enzyme. As a result, TGase treatment showed the least effect on sample 4.3. Moving away from the pI, the proteins are more soluble and flexible due to the increase in surface charge, providing a necessary condition

for the enzyme to come into contact. Thus, TGase treatment showed better performance on sample 7.0, 6.0 and 5.0. The positive effect of TGase treatment on PPIs functionality can be explained based on the fact that TGase linked protein molecules into bigger aggregates via covalent bond formation. Those big protein molecules provided more flexible structures that increased the ability to form layers around the oil/liquid and air/liquid interface with greater coverage. Consequently, the functionalities of TGase–treated PPIs were improved.

Based on results from SDS–PAGE, RVA and functionality tests, it was clear that TGase treatment at pH 6.0 gave more desired viscosity behavior and better functionalities in PPI compared to that at pH 7.0. Thus, pH 6.0 was chosen as the optimal pH level for TGase in PPI. This value was slight lower than values found in literature for legume in general. A pH value of 7.5 had been used as an optimal pH level for TGase treatment in phaseolus (*Phaseolus vulgaris*), pigeon pea (*Cajanus cajan*), cow pea (*Vigna unguiculata* L.) and hyacinths bean (*Dolichos hyacinth* L.) (Ali et al. 2010; Ahmed et al. 2010). Meanwhile, a pH value of 7.0 was used for PPI and fish protein hydrolysate mixture (Hu et al. 2010). However, the results proved that pH 6.0 was the optimal pH for TGase treatment under the specific extraction protocol that was used to extract yellow pea protein and was very close to the literature value for pea protein treatment, i.e. pH of 6.5 (Ribotta et al. 2012).

3. PEA PROTEIN EXTRACTS CHARACTERIZATION

3.1. Protein content

Four samples including freeze–dried control sample with final pH of 4.3 (CF 4.3), free dried TGase–treated sample with final pH of 4.3 (TF 4.3), spray–dried control

sample (CS), and spray–dried TGase–treated sample (TS) were tested for protein content using the combustion method. The result indicated that freeze–dried samples had significantly higher protein content compared to spray–dried samples. The difference in protein content between freeze–dried samples and spray–dried samples ranged from 54.8% to 63.6%. The protein contents for CF 4.3 and TF 4.3 were 88.3 ± 0.02% and 88.6 ± 0.08%, respectively. Meanwhile, protein contents for CS and TS were 57.1 ± 0.20% and 54.2 ± 0.46%, respectively (Table 18).

Table 18. Protein contents of different samples prepared by different extraction methods.

Sample*	Protein content
CF 4.3	88.3 ± 0.02%
TF 4.3	88.6 ± 0.08%
CS 7.0	57.1 ± 0.20%
TS 7.0	54.2 ± 0.46%
LS**	61.1 ± 0.11%

* CF 4.3: free dried control sample with final pH of 4.3, TF 4.3: free dried TGase–treated sample with final pH of 4.3, CS: spray–dried control sample with final pH of 7.0, TS: spray–dried TGase–treated sample with final pH of 7.0.
** pea protein extraction using method proposed by Bildstein et al. (2008).

This result is reasonable as freeze–dried samples had undergone an extra step during purification to remove the excess amount of soluble starches, resulting in higher purity of protein in final products. There was not much difference in protein content between the control and TGase–treated samples. Slightly higher protein content in a TGase–treated sample, i.e. 88.6 ± 0.08% vs. 88.3 ± 0.02%, might be the result of cross–linking between smaller soluble protein molecules to form bigger polymeric molecules that are less soluble than original small proteins and the TGase itself. Compared to literature value of other PPIs, our values are higher than those extracted by salt extraction, i.e. 81.1% (Karaca et al. 2011), equal to those extracted by ultrafiltration

(Vose 1980) and isoelectric precipitation (Karaca et al. 2011), i.e. 89.5% and 88.8%, respectively, but slightly lower than values of PPI extracted by acid extraction, i.e. 91.9% (Vose 1980). In general, values of different extraction methods are very similar, except for that of the salt extraction method. The slight difference in values between methods could come from the differences in raw material (i.e. varieties, growing location, protein content, moisture, and particle size), equipment, extraction conditions, and handling.

In contrast, spray–dried samples did not undergo the extra purification step, i.e. second centrifugation. The whole supernatants after enzyme treatments to degrade soluble starches were used in the spray drying step, leading to more carbohydrate components present in final products. This resulted in the reduction of protein percentage to less than 60% (Table 18). These samples, therefore, are considered pea protein concentrates (PPCs). Theoretically, there should not be any difference in protein content between CS 7.0 and TS 7.0 due to the fact that no other discard step was carried out after the first centrifugation. However, the result showed the TS 7.0 was approximately 3% lower than CS 7.0 (Table 18). The reason for the reduction is not clear but changes in extraction protocol, i.e. the use of additional enzymes amyloglucosidase and α–Amylase, temperature changing during extraction to optimize different enzymes' activities, may be factors that affect the protein results. No literature value was found for pea protein extraction prepared using the spray drying method but compared to other legumes studied by Bildstein et al. (2008), our spray–dried sample had a higher protein value. Repeating the literature method proposed by Bildstein et al. (2008) on yellow pea resulted in a higher protein value 61.1 ± 0.11%, which was very comparable to values of other

legumes using the same method, i.e. 50.3 ± 0.6% for lentils and 49.2 ± 0.05% for white bean (Bildstein et al. 2008).

Although protein contents of spray–dried samples were lower than those of freeze–dried samples, their recovery rate was probably higher than those of freeze–dried samples. Protein precipitation of supernatant, obtained from centrifugation, using 35% acetone revealed that at least 6.2% of total protein (db) had not been precipitated using the isoelectric precipitation at pH 4.3. Therefore, freeze drying method had at least 6.2% lower in protein yield compared to that of spray drying method due to the loss of soluble proteins in discard supernatant in freeze drying method.

3.2. Protein fractionation

Fractionation of pea protein isolates was carried out using SDS–PAGE method. CF 4.3 and TF 4.3 were two samples characterized along with a commercial PPI. The 12% non–reducing gel showed that CF 4.3 sample, i.e. control sample, did not possess any protein fraction bigger than 100,000 Da as no protein band was found in the region higher than 116,250 Da (molecular weight of β–galactosidase). Most protein bands were in the range of 45,000 Da to 66,200 Da (Figure 16). Only, small amounts of high molecular weight protein, i.e. bands at positions of approximately 100,000 Da, and low molecular weight protein, i.e. protein bands between 31,000 Da to 45,000 Da positions, were found in CF 4.3. Very faint bands found in region lower than 31,000 Da indicated the limited quantity of very small molecular weight protein in the control.

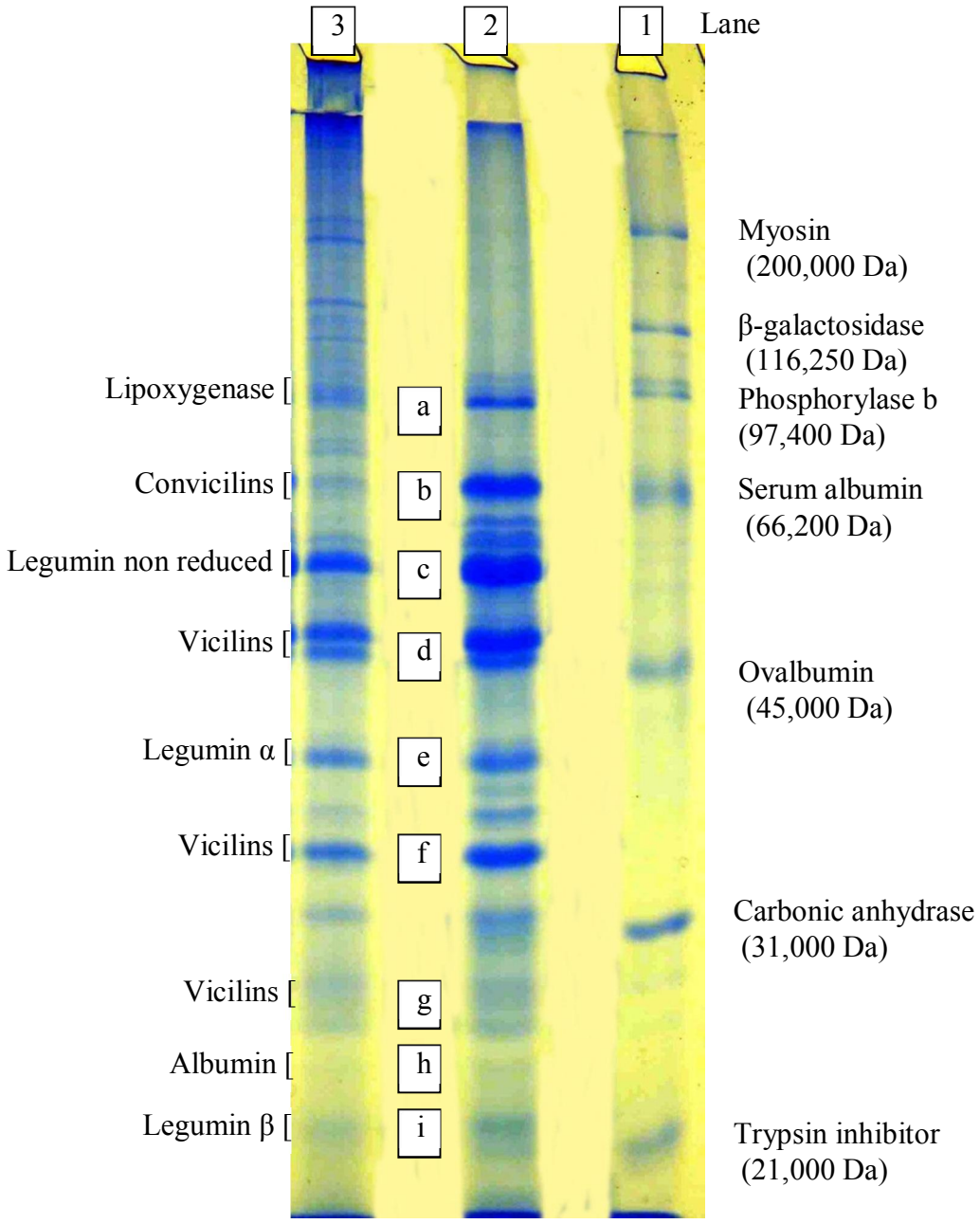


Figure 16. Protein fractions in TGase–treated pea protein isolates separated by 12% non–reducing SDS PAGE.
From right to left: 1. Standard; 2. CF4.3 (freeze–dried control PPI with final pH of 4.3); 3. TF4.3 (freeze–dried TGase–treated PPI with final pH of 4.3). Lower case letters, i.e. a, b, c, d, e, f, g, h, and i, indicate protein band positions.

The result of CF4.3 sample was in agreement with the literature (Barac et al. 2010) where the PPI on non–reducing gel had the same protein profile. Band at position

“a” represented lipoxygenases, position “b” represented convicilins, position “c” was legumin non reduced fraction, positions “d”, “f”, and “g” represented vicilins fractions, position “e” and “i” was legumin α and legumin β, respectively (Barac et al. 2010). The result also agreed with the fact that the major pea storage proteins are globulins, i.e. legumins (11S), vicilins (7S), and convicilins (Barac et al. 2010). The legumin was a hexamer with six subunit pairs that interact noncovalently (Barac et al. 2010). Each subunit pair consists of two subunits linked together via a single disulfide bond (Gueguen and Barbot 1988). These pairs are called legumin non–reduced. Since six subunit pairs do not link covalently, they were disassociated during sample preparation and presented as legumin non–reduced fraction in the non–reducing gel. Also, different legumin polypeptides was identified, e.g. 4–5 acidic (α) and 5–6 basic (β) polypeptides, due to the presence of a number of legumin precursors originating from several gene families (Heng et al. 2004). They were named legumin α and legumin β (Figure 16). Albumins are the second major storage proteins in pea (Gueguen and Barot 1988; Rao et al. 1989; Swanson 1990). However, only one protein band at position “h”, approximate 25,000 Da, was found to represent albumin fraction. This was in agreement with literature value where the main pea albumin was a dimmer of two homogenous subunits of approximate 25,000 Da (Croy et al. 1984, Rao et al. 1989). The other minor albumin of 6,000 Da (Rao et al. 1989) was not found and probably already moved off the gel.

However, the protein profile was significantly changed in the TF 4.3 sample. TF 4.3 had fewer proteins in all regions from 200,000 Da down to 21,000 Da. Also the lack of protein bands in region of 45,000 Da to 66,200 Da, i.e. legumin non–reduced and vicilin fractions, and the albumin fractions, i.e. position “h”, suggested that these

fractions were involved in the TGase catalyzed reaction to form high molecular weight protein molecules. Again, the result was in agreement with literature where albumins and globulins were found to be the main substrates for TGase catalyzed reaction (Macro et al. 2007). The newly formed proteins contained two groups: very large molecular weight protein molecules that can be referred to as protein aggregates and large molecular weight proteins. The protein aggregates were too big to travel through the gel and stuck on the well and the stacking gel. In contrast, the large molecular weight protein can travel through the stacking gel and formed bands on region between 116,250 Da to 200,000 Da (Figure 16). Those bands were faint and narrow, suggesting these proteins were not the main product of TGase reaction.

Protein fractionation was carried out on a reducing gel and the result was not different from that of non–reducing gel. Again, polymeric protein formed by TGase catalyzed reaction was stable and was not degraded by the reducing agent. Only a smaller change was found where proteins at position "a", i.e. legumin non–reduced, were disassociated into smaller protein subunits that traveled to the position "b", approximately 40,000 Da, and position "c", approximately 20,000 Da (Figure 17). This agreed with literature as the legumin non–reduced consists of two subunits linked together via a single disulfide bond (Gueguen and Barbot 1988). This disulfide bond was cleaved by the reducing agent, leading to the formation of the two subunits at positions "b" and "c". Other than that, other protein bands reflected single–unit fractions.

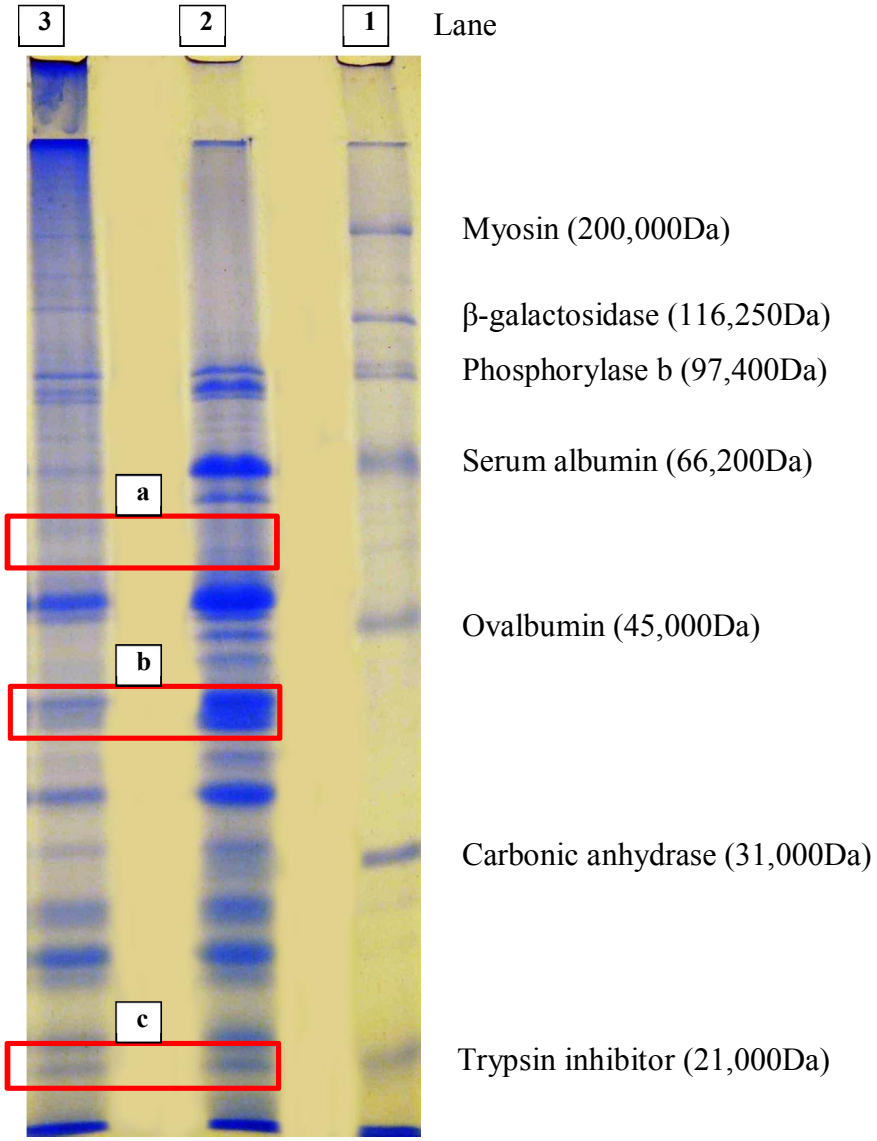


Figure 17. Protein fractions in TGase–treated pea protein isolates separated by 12% reducing SDS PAGE.
From right to left: 1. Standard; 2. CF4.3 (freeze–dried control PPI with final pH of 4.3); 3. TF4.3 (freeze–dried TGase–treated PPI with final pH of 4.3).Normal letters, i.e. a, b, c indicate protein band positions.

The 12% gel showed a better resolution compared to the 8% gel in both reducing and non–reducing tests, suggesting that pea proteins did not contain a high level of high molecular weight protein molecules except TGase–treated sample, i.e. TF 4.3 (Figure 18). An 8% gel was run with the purpose of separating the polymeric protein molecules

formed by TGase, but was not successful (Figure 18). This means the newly formed polymeric proteins were very large and consisted of more than three subunits. The substrates for TGase catalyzed reaction were protein molecules with molecular weight in between ~ 50,000 Da to ~ 100,000 Da as discussed in "polymerized protein formation" section. The dimers if formed should have molecular weight of less than 200,000 Da and would be present on the gels as myosin due to similar molecular weight, i.e. 200,000 Da. This complex was not detected on both the 12% and 8% gels. Again, this supported the hypothesis of the formation of protein aggregates with more than three protein molecules in TGase catalyzed reaction.

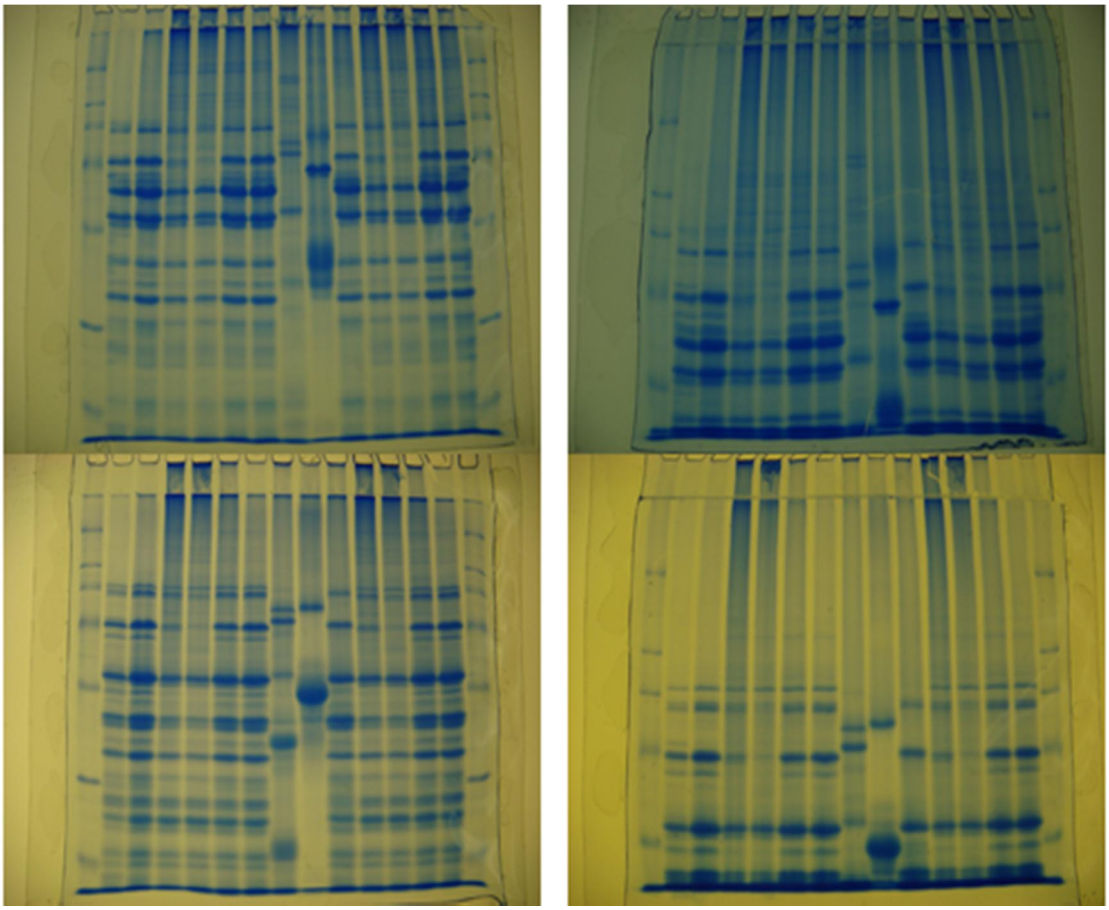


Figure 18. Resolution comparison between 8% and 12% SDS–PAGE gels.
Top left: 12% non–reducing gel, top right: 8% non–reducing gel, bottom left: 12% reducing gel, bottom right: 8% reducing gel.

Gel results for the commercial yellow PPI showed a slight difference in protein bands compared to our control PPI, i.e. control, especially at high molecular weight and low molecular weight regions. In the control sample, there was a cluster of three different protein bands at approximately 100,000 Da whereas there was only one band found in the commercial PPI sample. Similar observations were found in low molecular weight region where there was another cluster of several bands in the region with molecular weight smaller than 31,000 Da appearing in the control but not in the commercial sample. The results from the reducing gels showed a much different profile suggesting that protein composition in the PPI was different from that in the commercial PPI sample and that the prepared PPI had a wider protein molecular weight range.

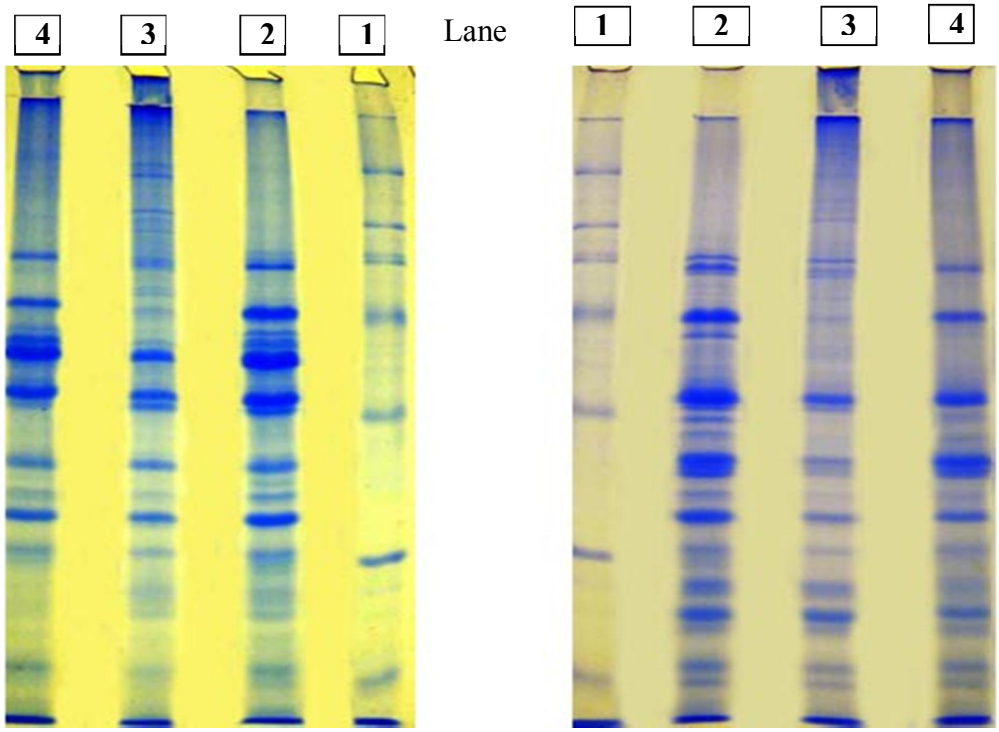


Figure 19. Protein profile comparison between PPIs.
Left: non–reducing gel, right: reducing gel. 1. Standard, 2. Control PPI, 3. TGase–treated PPI, 4. Commercial PPI.

3.3. Total starch content

The total starch results (Table 19) indicated that very low amounts of total soluble starch had been present in both freeze–dried and spray–dried samples. This means that the foaming, emulsifying and gelling functionalities were mainly a result of protein component in the final pea product and the effect of starch on functionalities had been minimized.

The TF 4.3 had a higher amount of starch compared to CF 4.3. In general, the control sample had approximately 60% lower soluble starch content compared to TGase–treated samples, i.e. 0.15 ± 0.02% vs. 0.36 ± 0.04% (Table 19). The hypothesis was proposed in which the TGase treatment, with primary purpose of increasing the size of protein molecules, also created an entrapment effect where soluble starches were retained in the newly formed protein aggregate. During the second centrifugation, these protein aggregates traveled to the bottom of the centrifuge tube along with the trapped soluble starches. In general, the use of TGase not only modified the protein profile but also created an entrapping effect that slightly changed the carbohydrate profile of PPI towards the increase in high molecular weight carbohydrates, i.e. soluble starches. No literature was found on entrapping effect of cross–linked pea proteins by TGase but the entrapment of bovine serum albumin (BSA) on a gel formed by soy proteins cross–linked by genipin was reported (Song and Zang 2009). The authors reported that increasing level of cross–linking reduced the amount of BSA adsorbed onto the gel matric but also reduced the rate of BSA release. It meant that cross–linking helps better retain BSA molecules inside the matrix once they are entrapped.

Table 19. Total starch content in different pea protein extracts.

Sample*	Total starch content**, % (db)
CF 4.3	0.15 ± 0.02^{a}
TF 4.3	0.36 ± 0.04^{b}
CS 7.0	0.15 ± 0.03^{a}
TS 7.0	0.28 ± 0.10^{b}

* CF 4.3: free dried control sample with final pH of 4.3, TF 4.3: free dried TGase–treated sample with final pH of 4.3, CS 7.0: spray–dried control sample with final pH of 7.0, and TS: spray–dried TGase–treated sample with final pH of 7.0.
** Samples with different letters indicate significant differences ($p < 0.05$).

Similar results were found on spray–dried samples where TS 7.0 had a higher starch content compared to CS 7.0, i.e. 0.28 ± 0.10% vs. 0.15 ± 0.03% (Table 19). In general, control samples of both freeze dry and spray dry methods had 50 – 60% lower soluble starch content compared to TGase–treated samples. Again, one possible explanation could be the entrapment effect that TGase created on the pea slurry. Since the TGase treatment was carried out prior to the amyloglucosidase treatment, the formation of big protein aggregates by TGase catalyzed reaction might entrap a certain amount of soluble starches remaining after α–Amylase treatment. This prevented amyloglucosidases contact with their substrates and to degrade them into sugars and short chain oligosaccharides. Consequently, these trapped soluble starches went into the final protein products, contributing to the high level of total starch content of such products.

Total starch was determined instead of total carbohydrate, i.e. including starch, oligosaccharides and sugars. Oligosaccharides and sugars had a minor impact on gelling due to the fact that they do not meet a minimum critical chain length necessary for the cooperative nature of the interaction causing gel formation, which typically is in the range 15 to 20 residues (Whistler 1973). Polysaccharide also affects emulsifying property

of protein extracts. When polysaccharide concentration is sufficiently high, they form a three–dimensional network through intermolecular entanglements that entraps the oil droplets and effective inhibits their movement, therefore improving the stability of the emulsion system (Wang and Cui 2005). Thus, it is more important to know the total starch content, which possibly had a bigger impact on the functionalities of final pea protein products, rather than the total carbohydrate.

3.4. Other characters

3.4.1. Moisture

Freeze–dried samples had moisture levels ranging from 1.6% to 1.8% whereas the spray–dried samples had moisture levels ranging from 3.4% to 4.3%. Result of all samples showed that freeze–dried samples had relatively lower moisture compared to spray–dried samples. This was due to the nature of two drying methods. Freeze drying tends to produce low moisture levels in samples due to a prolong drying time, which can last from several hours to several days under vacuum condition. Depending drying time, moisture can drop to as low as 0.2%. Several factors affecting drying time such as amount of sample to be dried, water content of samples, and surface area.

In contrast, spray drying method was relatively quick. Depending on the capacity of the spray drier, the drying time could vary from several seconds to a minute. A typical time ranges from 1 to 20 s (Brennan 2006). Due to this short time, along with the limitation in input temperature to maintain product organoleptic characteristics, spray–dried samples usually have moisture levels of several percent.

3.4.2. Ash content

Mineral contents of freeze–dried samples were found to be lower than those of spray–dried samples. Both ash contents for CF 4.3 and TF 4.3 were 2.7 ± 0.0% whereas ash contents for CS 7.0 and TS 7.0 were 7.7 ± 0.2% and 7.3 ± 0.1%, respectively. These values were reasonable as most minerals in pea slurry were in soluble forms and removed from the supernatant during the second centrifugation, leading to the low ash content in freeze–dried samples. The significant high amount of ash in spray–dried samples came from two factors. First, the whole supernatant was used in the process, thus no ash was removed. Second, using sodium hydroxide to extract protein and hydrochloric acid to adjust the pH of the pea supernatant added an extra amount of mineral to the final product, i.e. sodium chloride. The two factors brought the ash content in the spray–dried samples to approximately three times higher than ash content in the original pea flour, i.e. 2.5 ± 0.0%.

3.4.3. Particle size

Freeze–dried samples tend to form big clumps when dry. Therefore, it is necessary to have an additional step to grind these clumps into the desired particle size (Figure 20). Spray–dried samples did not need further grinding step as they were already in powder form when leaving the spray dryer (Figure 20).

Figure 20. Visual comparison between freeze–dried and spray–dried samples.

4. PEA PROTEIN EXTRACT FUNCTIONALITIES

4.1. Foaming properties

Foaming properties included foaming capacity and foaming stability summarized in Table 20. Foaming capacity result showed that freeze–dried samples had significantly lower values compared to spray–dried samples. In contrast, foaming stability of freeze–dried samples was higher than those of spray–dried samples (Table 20).

Table 20. Foaming properties of different pea protein products.

Sample*	Foaming capacity (%)	Foam volume, mL at 30 mins	Foaming stability (%)
CF 4.3	4.5 ± 0.0	1	11.1 ± 0.0
TF 4.3	3.3 ± 0. 3	1.3	19.8 ± 7.7
CS 7.0	20.3 ± 2.5	1	2.5 ± 0.3
TS 7.0	23.2 ± 0.6	1	2.2 ± 0.1
LS 7.0	26.7 ± 2.6	26.9	47.1 ± 8.7
Commercial PPI	22.2 ± 0.3	0.7	1.5 ± 1.3
Egg albumin	18.3 ± 1.6	33	90.1 ± 2.6
FP**	80[a]	138	86.3[b]
LSD***	2.2	n/a	8.2

* CF 4.3: free dried control sample with final pH of 4.3, TF 4.3: free dried TGase–treated sample with final pH of 4.3, CS 7.0: spray–dried control sample with final pH of 7.0, TS 7.0: spray–dried TGase–treated sample with final pH of 7.0, LS 7.0: PPC prepared by method proposed by Bildstein et al. (2008).
** foaming protein, extracted from second supernatant using 35% acetone solution. [a,b] did not have enough sample to run test in triplicate.
*** LSD calculation did not included FP data.

It could be the protein solubility that caused the difference in foaming functionalities between freeze–dried– and spray–dried samples. Good solubility is one of the requirements for a protein to be an ideal foam–forming and foam–stabilizing agent (Belitz et al. 2009). Periago et al. (1998) found that foaming capacity of pea flour was reduced as the protein solubility decreased. The freeze–dried samples with a final pH of 4.3, which was their pI, had the least protein solubility when their reconstituted slurries were tested for foaming. Meanwhile, the spray–dried samples had a final pH of 7.0 and thus had more soluble proteins. Besides, second centrifugation removed a certain amount of soluble protein from freeze–dried samples, which further lessen the amount of active soluble protein in freeze–dried samples compared to spray–dried samples. Consequently,

the foaming capacity of spray–dried samples was higher than that of freeze–dried samples.

Foaming capacities were comparable among spray–dried samples, i.e. CS 7.0, TS 7.0, LS 7.0 and commercial PPI (Table 20). Foaming protein (FP) was found to have highest values in both foaming capacity and foaming stability in all pea samples. The FP did not precipitate at pI 4.3 but was collected using organic solvent extraction technique, i.e. 35% acetone solution. The high foaming capacity of this protein may contribute to a significantly high foaming capacity of spray–dried samples compared to those of freeze–dried samples. Since spray–dried samples contained FP, due to the fact that the whole slurry was spray–dried, they possessed high foaming capacities. Freeze–dried samples did not contain FP, thus resulting in low foaming capacities.

The magnitudes of foam volumes after 30 minute storage were almost the same for all samples, i.e. 0.7 mL to 1.3 mL (Table 20), but the foaming stabilities of spray–dried samples except LS 7.0 sample were significantly lower compared to those of freeze–dried samples. There could be two reasons for this difference. First, the foaming stabilities of freeze–dried samples expressed in percentage were substantially higher than those of TS 7.0, CS 7.0, and commercial PPI due to the small zero time volumes of freeze–dried samples, i.e. 6.7 mL and 9 mL for TF4.3 and CF4.3, respectively. The second reason is the difference in protein – protein interactions that occur during testing of freeze–dried and spray–dried samples. The final pH of 4.3 in freeze–dried samples promotes the protein – protein associations via hydrophobic interactions, which reduce the protein solubility and consequently the foaming capacity of freeze–dried samples but

increase their foaming stability due to stronger cross–linkage of protein molecules at interface (Fennema 1997; Belitz et al 2009).

The presence of FP in the spray–dried samples, i.e. CS 7.0, TS 7.0, cannot improve the foaming stability as seen in LS 7.0 sample. The LS 7.0 showed a significantly high level of foaming stability, i.e. 47.1%, compared to other spray–dried samples, which had foaming stability levels ranging from 1.5% to 2.5% (Table 20). On the other hand, the protein percentage in final extracts was close, i.e. 57.1%, 54.2% and 61.1% for CS 7.0, TS 7.0 and LS 7.0, respectively. Thus, the answer for this remained unclear. However, it might be differences in extracting conditions, i.e. extracting pH, precipitating pH, enzyme treatment conditions, that caused changes in protein nature and protein profile, leading the higher foaming stability in the LS 7.0 compared to those of CS 7.0 and TS 7.0 samples.

TGase treatment did not change the foaming stability but slightly improved the foaming capacity of spray–dried samples. In contrast, TGase treatment improved the foaming stability but slightly reduced the foaming capacity of freeze–dried samples. The slight decrease in foaming capacity of freeze–dried samples did not really reflect the effect of TGase treatment. As TGase cross–linked protein molecules together to form bigger aggregates, the solubility of protein decreased. The insolubility of protein was further aided with the low final pH of freeze–dried samples, i.e. pI, causing a reduction in foaming capacity of freeze–dried samples. However, this increased the foaming stability as previous discussed. In general, the results agreed with literature findings where TGase treatment was found to improve foaming properties of protein isolates from pigeon pea and hyacinth bean (Ali et al. 2010).

Literature values for foaming properties were not consistent. Bildstein et al. (2008) reported the foaming fraction of 16% for a commercial pea protein concentrate, which was lower than those of the spray–dried samples, i.e. approximately 20.3%, 23,2%, and 26.7% for CS 7.0, TS 7.0, and LS 7.0, respectively, but higher than those of freeze–dried samples, i.e. 3.4% and 4.5% for CF 4.3 and TF 4.3, respectively. The spray–dried samples' foaming fraction was also higher than that of a pea protein reported by Fernandez – Quintela et al. (1997), i.e. 16%, but their foaming stabilities were lower than that of a pea protein, i.e. 94%. Vose (1980) also found that protein isolates from smooth–seeded yellow pea (*Pisum sativum* L. cv. Trapper) prepared using ultrafiltration had superior foaming properties in comparison with skim milk powder, soy protein isolate (SPI) and wheat flour. Variations in the results could be due to differences in the protein purity of the samples studied as well as the specific conditions used for the foaming tests (Boye et al. 2010).

4.2. Emulsifying properties

Emulsifying properties consisted of emulsion capacity and emulsion stability (Table 21).

In general, freeze–dried samples had lower emulsion capacity in comparison with spray–dried samples. The highest values were found in the LS 7.0 and FP samples whereas lowest value was found in the TS 7.0 sample. Between the two freeze–dried samples, emulsion capacity was not much different, i.e. less than 5% difference. However, between the CS 7.0 and TS 7.0 samples, the difference was approximately 28%. All samples prepared in the research, i.e. CF 4.3, TF 4.3, CS 7.0, and TS 7.0, had lower emulsion capacities compared to that of the egg albumin and much lower than

those of the LS 7.0 and FP. The CS 7.0 sample had a comparable emulsion capacity to the commercial PPI, which was prepared using spray drying.

Table 21. Emulsifying properties of different pea protein products.

Sample*	Emulsion capacity g oil/g extract	Emulsion stability %
CF 4.3	268.5 ± 7.64	45.8 ± 1.0
TF 4.3	280.4 ± 9.78	50.6 ± 4.1
CS 7.0	315.8 ± 9.62	44.1 ± 1.0
TS 7.0	225.8 ± 1.33	43.5 ± 1.0
LS 7.0	515.3 ± 3.35	52.4 ± 1.0
Commercial PPI	317.5 ± 21.16	48.2 ± 1.8
Egg albumin	369.9 ± 26.11	51.8 ± 0.0
FP**	521.6[a]	46.4[b]
LSD***	23.8	3.6

* CF 4.3: free dried control sample with final pH of 4.3, TF 4.3: free dried TGase–treated sample with final pH of 4.3, CS 7.0: spray–dried control sample, TS 7.0: spray–dried TGase–treated sample, LS 7.0: PPC prepared by method proposed by Bildstein et al. (2008).
** foaming protein, extracted from second supernatant using 35% acetone solution. [a,b] did not have enough sample to run test in triplicate.
*** LSD calculation did not included FP data.

The lower values found in freeze–dried samples compared to those of spray–dried samples were expectable due to the poor solubility of freeze–dried PPIs. This was in agreement with previous reports which showed a positive relationship between protein solubility and emulsification capacity (Fuhrmeister and Meuser 2003; Barac et al. 2010). Again, protein solubility is an important factor of a good emulsifier (Belitz et al. 2009). Once the protein solubility increased, the emulsion capacity was significantly improved. If considering emulsion capacity per g of protein instead of per g of extract, the spray–dried samples had 18% to 44% higher emulsion capacity compared to freeze–dried samples. The emulsion capacity of the CS 7.0 sample, i.e. equivalent to 527 g oil/ g

protein, was higher compared to literature values of pea protein isolates whereas those of CF 4.3, TF 4.3, and TS 7.0 samples were lower. Karaca et al. (2011) reported the values of 477.78 g oil/ g protein and 484.45 g oil/g protein for pea protein isolates prepared by isoelectric precipitation and salt extraction methods, respectively.

TGase treatment caused a slight increase in emulsion capacity in freeze–dried samples but a great reduction in spray–dried samples. The result showed that TGase treatment reduced the emulsion capacity of spray–dried samples to ~ 28%. In general, an effect of TGase treatment on freeze–dried samples was in agreement with literature but the effect of TGase treatment on spray–dried samples was opposite to the literature findings. It was reported that TGase treatment on protein isolates from pigeon pea and hyacinth bean greatly improved their emulsifying properties (Ali et al. 2010). The answer remains unclear as to why there were two complete different trends for freeze–dried samples and spray–dried samples as shown in the results.

There was the same observation on emulsion stability as seen in foaming stability in which TGase treatment did not cause significant changes in the emulsion stability of spray–dried samples but significantly improved those of freeze–dried samples. Again, TGase cross–linked protein molecules together to form bigger aggregates which precipitate out of the solution. The low final pH of freeze–dried samples, i.e. pI, further induces the protein – protein interaction via hydrophobic interactions that strengthen protein film surrounding oil droplets, leading to the increase in emulsion stability. However, result only showed slight improvements, suggesting that protein solubility did not greatly associate with emulsion stability. This was agreed with literature findings where Barac et al. (2010) reported a non–significant correlation between emulsion

stability and solubility of six different pea (*Pisum sativum*) genotypes. The effect of TGase treatment on emulsion stability of yellow pea was similar to those of pigeon pea and hyacinth bean as reported by Ali et al. (2010).

4.3. Gelling properties

Gelling capacity was tested using the method proposed by Bildstein et al. (2008). The result showed that most of the sample prepared in the research did not gel. Only the LS 7.0 sample, which was prepared using method proposed by Bildstein et al. (2008) formed a weak gel (Table 22). The spray–dried samples had better water holding capacity, as less released water was obtained from gels, compared to the freeze–dried samples.

Table 22. Gelling properties of different pea protein extracts.

Sample*	No centrifuge		Centrifuge at 3,000 rpm in 5 mins	
	V_{H2O}, mL	Gelling	V_{H2O}, mL	Gelling
CF 4.3	3.5	No	3.5	n/a
TF 4.3	3.5	No	3.5	n/a
CS 7.0	1.0	No	4.3	n/a
TS 7.0	1.0	No	3.2	n/a
LS 7.0	0	Weak	1.0	n/a

* CF 4.3: free dried control sample with final pH of 4.3, TF 4.3: free dried TGase–treated sample with final pH of 4.3, CS 7.0: spray–dried control sample, TS 7.0: spray–dried TGase–treated sample, LS 7.0: PPC prepared by method proposed by Bildstein et al. (2008).

The TGase treatment did improve viscosity of the pea protein slurries as indicated in RVA results but it did not help in improving gelling properties of pea protein extracts as no difference in gel formation was found between non–treated and treated samples. TGase treatment also did not improve the water holding capacity of pea protein extracts.

The volume of water released after storage, V_{H2O}, at both conditions, i.e. no centrifugation and centrifugation at 3,000 rpm in 5 mins, supported this conclusion (Table 22). This result disagrees with literature findings where TGase treatment was found to improve the gel strength of PPIs (Shand et al. 2008; Sun and Arntfield 2011a). However, in these studies, the dynamic rheological properties of the PPIs were measured only via the two factors of G' (elastic modulus) and G" (viscosity modulus) but not the real gel formation. Thus, it is difficult to compare results from two different approaches. As the RVA showed an increase in viscosity of TGase–treated samples compared to non–treated samples, the PPIs in this research may show the same result as literature if the same method of dynamic rheological measurement was used. A difference in varieties used could be an additional explanation for the difference. O'Kane et al. (2005) reported that different pea cultivars performed differently on gelation behavior. Five cultivars including Solara, Supra, Classic, Finale, and Escape were grown under the same conditions but only Solara formed a strong gel. Supra and Classic were only able to form weaker gels whereas Finale and Escape were unable to form self–supporting gels (O'Kane et al. 2005). Since different studies used different varieties/cultivars, the comparison becomes difficult.

The result also indicated an important role of extracting conditions on gelling capacity of pea protein extracts. The LS 7.0 was able to form a weak gel whereas others did not, suggesting different extraction protocols gave different gelling results even though the same raw material was used during the extraction.

4.4. Effect of pH on pea protein isolates' functionality

Previous functionalities tests done on freeze–dried samples showed a lower result compared to those of spray–dried samples and literature values. One of the reasons was proposed to be the poor protein solubility at pH 4.3. Therefore, the effect of final pH on pea protein functionalities was also investigated. Two final pH levels were tested including a set of samples with final pH of 4.3, i.e. CF 4.3 and TF 4.3, and a set of samples with final pH of 7.0, i.e. CF 7.0 and TF 7.0.

The result showed a significant improvement in almost all functionalities except the emulsion stability when final pH value raised from 4.3 to 7.0 (Table 23). In general, foaming capacity improved ~330% – 580%, foaming stability increased ~490% to 2,000%, emulsion capacity improved ~170% to ~260%, meanwhile emulsion stability was not affected.

Table 23. pH effects on foaming and emulsion properties of different PPIs.

Sample*	Foaming capacity (%)	Foaming stability (%)	Emulsion capacity (g oil/g PPI)	Emulsion stability (%)
CF 4.3	3.0 ± 0.5	12.2 ± 10.7	206.2 ± 2.36	44.1 ± 1.0
CF 7.0	17.3 ± 0.8	59.5 ± 3.8	545.3 ± 8.75	50.0 ± 0.0
TF 4.3	4.5 ± 0.9	3.3 ± 5.8	199.8 ± 6.63	45.2 ±2.7
TF 7.0	14.8 ± 2.0	66.5 ± 2.4	341.2 ± 7.83	40.5 ± 2.7
LSD	2.0	11.5	14.4	3.5

* CF 4.3: free dried control sample with final pH of 4.3, TF 4.3: free dried TGase–treated sample with final pH of 4.3, CF 7.0: free dried control sample with final pH of 7.0, TF 7.0: free dried TGase–treated sample with final pH of 7.0.

Again, this result agreed with literature where solubility was reported to be an important factor affecting foaming and emulsion properties of a protein (Belitz et al.

2009) but not significantly correlating to emulsion stability of a protein (Barac et al. 2010). All functionality results of PPIs with final pH of 7.0, i.e. CF 7.0 and TF 7.0, were comparable to literature values (Boye et al. 2010; Karaca et al. 2011).

5. APPLICATION IN REAL FOOD SYSTEMS

A preliminary research was done to verify the impact of final pH on performance of pea protein isolates on real food systems, i.e. cake system. The result showed that PPIs with final pH of 7.0 produced very sticky crumb and dense cakes whereas the PPIs with final pH of 4.3 produced much more acceptable cakes, indicating the PPIs with final pH of 4.3 showed a better performance in real food systems although performing poorer on functionalities tests. Thus, the PPIs with final pH of 4.3, i.e. TF4.3 and CF4.3, were chosen to be tested on cake and cookie systems.

Application in real food systems of PPIs was evaluated by two different ways including physical test and sensory evaluation. Results from both methods were combined to determine the possibility of the PPIs to be used as an egg replacer in cakes and cookies.

5.1. Physical tests

5.1.1. Cake

5.1.1.1. Cake height

The 20 cm diameter cakes were cut in half and measures were taken at different positions according to AACCI method 10–91. Result showed that egg formula had the highest center height, followed by TGase–treated PPI (TGase) and PPI formulas (Figure 21). Among the three formulas, TGase sample had the best cake–like shape with the

center point was the highest point among all measured points. Both egg and PPI samples did not produce a good shape due to either" a shadow indent" in the center of egg sample or a "flat–like shape" in PPI sample. None of the formulas produced a good symmetry cake but TGase was the best.

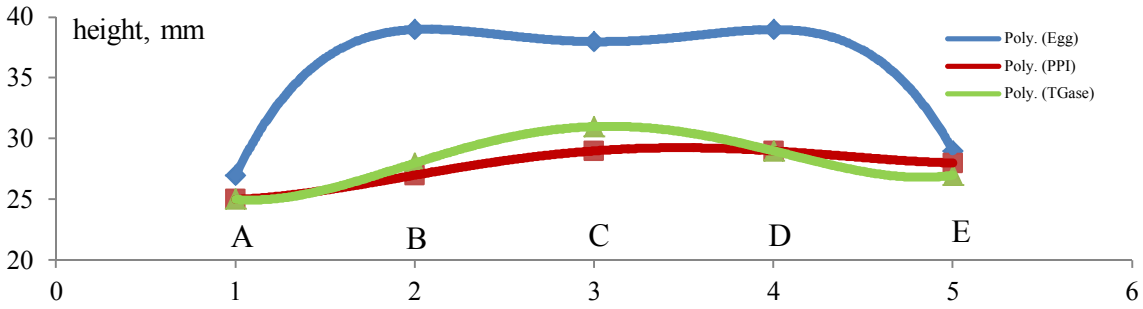


Figure 21. Cake shapes of different 20–cm–diameter cakes.
Values at each position were the average of two measurements.

In terms of cake volume, the egg formula had the greatest expansion whereas the PPI sample produced the lowest cake volume. This indicated that none of the pea formulas performed as comparable as the egg formula in term of cake volume. The TGase formula performed better than the PPI formula. Besides, TGase formula also showed a better cake symmetry compared to the PPI formula, suggesting TGase treatment improved the PPI's performance in cake system. Alp and Bilgicli (2008) reported the similar findings where the combination of TGase and low protein flour gave more puffed, symmetrical, and softer cake samples. No literature was found on effect of TGase–treated PPI on cake quality.

5.1.1.2. Cake texture

Cake texture was evaluated by 3 parameters including hardness, adhesiveness, and springiness (Figure 22). Hardness is the peak force (N) required to compress the cake

to 50% of its height. Adhesiveness represents work (J) required to pull the compressing plunger away from the samples. Springiness measures a height (mm) that the sample recovers between the end of the first cycle and the start of the second cycle.

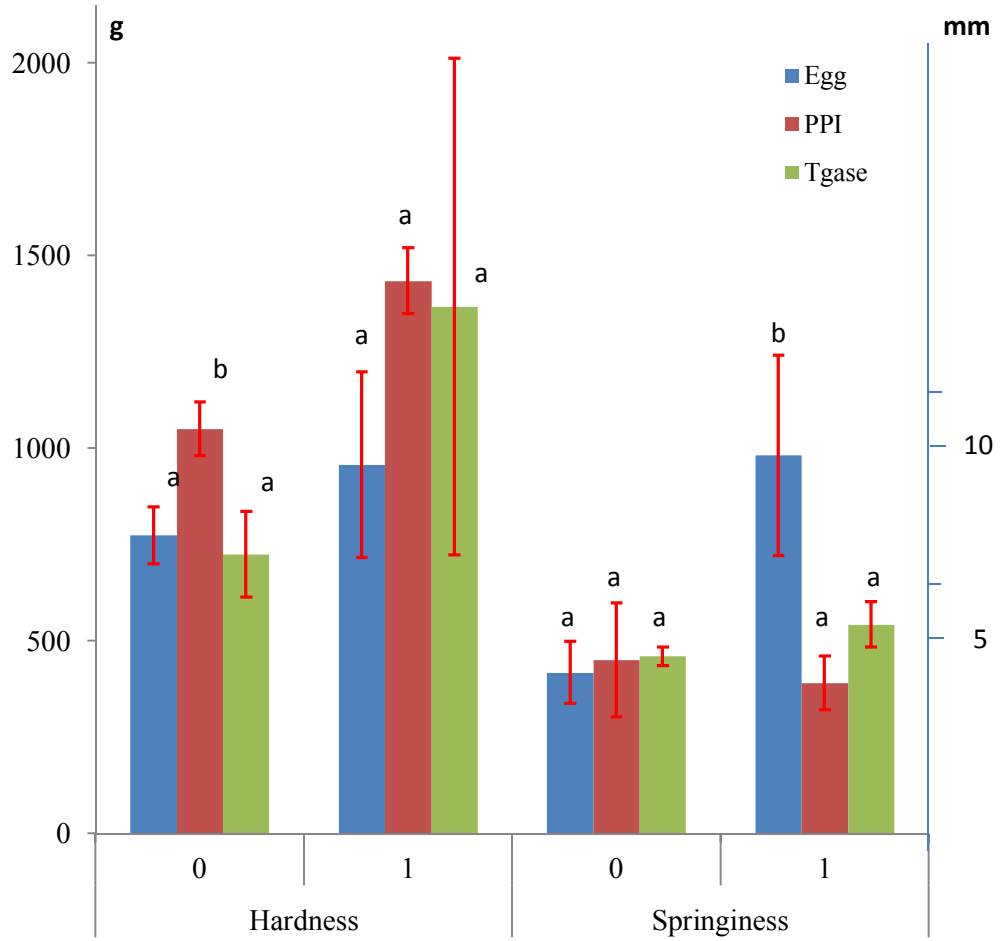


Figure 22. Hardness and springiness comparison between different cake samples. 0: day zero after cake baking. 1: day 1 after cake baking. Lower case letters indicate significant differences ($p < 0.05$). Bars indicate ± SD.

The hardness results showed that cake made with PPI, i.e. PPI sample, had the highest values on both day 0 and day 1 among all samples. Cake made with egg, i.e. egg sample, had a higher hardness than TGase on day 0 but was lower on day 1. However, the differences in hardness between egg and TGase samples are not significantly different in

both days (Figure 22). In contrast, PPI sample's hardness was significantly different from other samples in day 0 but not in day 1. The PPI formula produced the strongest cake texture based on the hardness measurements. However, if considering the result in correlation with the cake volume values, this high value actually came from a dense crumb due to the lowest cake volume of the PPI sample. Staling is another factor used to evaluate a performance of samples. An increase in hardness between day 0 and day 1 indicated staling of a sample. Among all samples, cakes made from egg showed the least change, i.e. 25% increase, in hardness followed by PPI (36%) and TGase (89%). Combining both results from hardness and staling, the egg sample had the better hardness properties compared to other samples as the staling process was delayed while having as strong texture as the TGase sample. The TGase sample performed comparable to the egg sample in hardness but not on staling.

Springiness is another important factor which measures the ability of a cake to return to its original size and shape after being compressed by an external force. The springiness was almost the same as day 0 but slightly changed on the day 1. The springiness of the egg sample increased while springiness remained unchanged for cake made with PPI and TGase. The significant increase in egg sample's springiness was proposed to be caused by longer time storage provided enough time for water to evenly distribute and obtain the equilibrium state within the cakes, resulting in better springiness compared to the day 0. This did not happen with both PPI and TGase samples, proposing both pea containing cakes obtain their stable state faster than the egg sample. Between two pea samples, the TGase samples showed a slightly better springiness compared to the PPI samples (Figure 22).

Adhesiveness was also tested but all of the samples had a zero adhesiveness result, indicating none of the cake produced a sticky crumb. Sticky cake crumb is usually not desired thus the result indicated that all samples performed well in this attribute.

The higher springiness value in association with higher hardness and lower adhesiveness indicates a better cake. Thus, the egg sample outperformed all pea samples in terms of physical tests. Between pea samples, the TGase sample was considered better than the PPI sample as it had better cake volume, cake symmetry, and other crumb characters. This agreed with literature where TGase was reported to improve cake symmetry, crumb softness and crumb volume if use in combination with low protein flour (Alp and Bilgicli 2008).

5.1.2. Cookie

Cookies used in physical test were drop cookies with an average weight of dough of 12 g/cookie. Cookie height and length were conducted using 8 cookies stacked or laid side–by–side (Table 24).

Table 24. Physical measurements* of cookies made with egg or pea proteins.

Formula	8 cookie height, mm	Cookie height average, mm	8 cookie length, mm	Cookie diameter average, mm
Egg	91 ± 2	11.4	383 ± 1	47.9
PPI	90 ± 4	11.3	383 ± 2	47.9
TGase	94 ± 3	11.8	382 ± 3	47.8

* Values were the average of three measurements ± standard deviation (SD).

Results from the physical tests showed that there was almost no difference between different samples in both cookie height and cookie length. TGase samples had a slightly better cookie height, followed by egg and PPI. In contrast, all three samples had

similar length values with the egg and PPI samples having approximately 0.3% higher cookie length compared to that of the TGase sample. The result was reasonable as the height and the length usually inversely related. The cookie height represented the ability of a cookie to resist to extension whereas the cookie length associated with its spreadability. So, if a cookie had a higher height value, its length/diameter is usually smaller. Consequently, TGase samples had a larger height but a shorter length meanwhile, egg and PPI samples had a longer length but a shorter height. The result did not show which sample performed better than the others as the differences in height and length were very small. Therefore, it can be concluded that both PPI and TGase performed as well as egg in the cookie system.

Physical tests in both cake and cookie systems indicated that there was no difference in performance of pea samples against egg samples in cookies but egg samples outperformed pea samples in cake. Thus both PPI and TGase can fully replace egg in some particular food systems, e.g. cookie, but can only partially replace egg in others, e.g. cake. The TGase performance was better than the PPI in cake in almost all aspects including cake volume, cake shape, cake symmetry, and crumb springiness except staling. Both PPI and TGase showed a lower staling resistant compared to egg. As both PPI and TGase–treated PPI used in cake had a final pH of 4.3, their solubility was limited, resulting in poor water holding capacity and consequently poor staling resistance. Preliminary experiments done on PPI and TGase samples with final pH of 7.0 showed that cake made from such pea products had a very sticky and dense crumb. Thus the pea products with final pH of 4.3 were used.

Physical test showed that pea extracts produced cake with less desirable characteristics, i.e. cake volume, crumb quality, compared to egg but it did not mean they produced unacceptable or poor quality cakes. Similarly, non–difference between pea extracts and egg performances in cookies does not mean that consumers will accept pea cookies. Only consumer acceptability test combining with physical tests could provide a proper answer. Thus, sensory evaluation was carried on both cookie and cake to further evaluate the possibility of using pea extracts as an egg replacer.

5.2. Sensory evaluation

5.2.1. Cake sensory

Appearance, flavor, texture, and overall acceptability were the 4 attributes tested in the cake sensory (Table 25). The first three attributes were independent characteristics of a sample whereas the last attribute, i.e. overall acceptability, was a dependent variable and usually affected by the first three.

Table 25. Sensory evaluation* of cake attributes of different cake formulas.

Formula***	Appearance**	Flavor**	Texture**	Overall acceptability**
Egg	5.97 ± 1.86^{b}	6.14 ± 1.79^{a}	4.93 ± 2.11^{b}	5.55 ± 1.33^{b}
PPI	7.22 ± 1.35^{a}	5.84 ± 1.99^{a}	6.25 ± 1.33^{a}	5.97 ± 1.88^{a}
TGase	7.01 ± 1.33^{a}	5.41 ± 1.99^{b}	6.09 ± 1.61^{a}	5.63 ± 1.79^{ab}

* Values were calculated on a 9–point hedonic scale.
** Samples with different letters indicate significant differences ($p < 0.05$).
*** Egg: cake made with egg, PPI: cake made with pea protein isolate, TGase: cake made with TGase–treated pea protein isolate.

Cake made with egg had the lowest acceptance from consumers in appearance and texture (Table 26), indicating a completely opposite result to the physical test where egg sample showed the best cake volume and crumb structure. This suggested that having

large cake volume and spongy crumb did not mean the egg sample would be more accepted than the pea samples. At the same level of added water, the egg with better functionalities, i.e. gelling, emulsifying, foaming, and water holding capacity, compared to pea protein extracts, was able to form larger crumb cells and held the cake batter better during baking, leading to the formation of spongier and drier crumb. In contrast, pea protein extracts with poorer functionalities were only able to create smaller volume cakes with moist and denser cake crumbs. Comments from panelists suggested that a cake crumb should be a little moist and should not too spongy. That was the reason why consumer neither liked nor disliked the egg samples' texture. Appearance and texture scores showed that there was no significant difference between the PPI and the TGase samples but the PPI had better scores than the TGase. Again, TGase had a better performance based on the physical test but consumer seemed to like the PPI more in both appearance and texture. The result suggested that the physical test and the sensory evaluation did not always agree with each other and therefore, combining results of these two tests was necessary.

However, the egg sample gained the best score in flavor although it was not significantly different from that of the PPI sample, implying pea extracts caused some unfavorable flavors in cake samples. Consumers provided some comments of "strange" aftertaste on pea samples and that might be the reason the pea samples received the lower score than the egg sample. Between the PPI and TGase samples, the TGase had significantly low score compared to the PPI. This suggested that TGase treatment caused a more severe off–taste problem. The "strange" aftertaste was proposed to be a consequence of two different causes. The low pH, i.e. pH 4.3, in pea extracts was

proposed to be the first reason causing an off–taste problem in cakes and cookies. No literature was found for cakes and cookies but similar findings were reported in milk. Porubcan and Vickers (2005) reported the effect of decreased pH on milk aftertaste that caused an increase in intensities of "sour" and "dairy sour" in milk products. However, this change only affected the aftertaste, but did not significantly affect hedonic rating (Porubcan and Vickers 2005). The cake results were similar to literature findings where a "strange" aftertaste was reported but no significant difference was found, i.e. between egg sample and the PPI sample. The second reason could be the presence of remaining pea flavor substances and newly formed substances in pea protein extracts. Consequently, those substances caused the unfavorable flavor in cake samples.

It was reasonable that egg gained the lowest score on overall acceptability. As mentioned, acceptability was a dependent variable whose score was determined by combination of all three independent variables which are appearance, texture, and flavor. Due to the lower score on two attributes of appearance and texture, the overall acceptability score of egg sample was reduced and significantly lower than those of the PPI samples. No difference was found between the TGase and PPI samples, although the latter showed a little better score.

In overall consideration, TGase performed better than PPI in cake application. It produced cakes with better physical properties and comparable sensorial evaluation. The aftertaste was the only issue that TGase needs to improve. In fact, the unfavorable aftertaste could be eliminated if purification step was used in the extraction process. Dialysis or "washing" are samples of purification process that are believed to reduce the amount of unwanted substances in final PPIs, thus improving the cake aftertaste.

It was clear that consumer test did not favor the egg over the pea samples but the physical result indicated a better performance of egg compared to the pea samples. This means the current TGase–treated PPI was not sufficient to replace egg in cake systems at least in physical test aspect. It is, therefore, suggested the combination of TGase treatment with other treatments to further improve pea protein functionalities, especially in gelling properties. One of the proposed treatments was the modification of protein with galactomanan through the Maillard reaction. It was reported that modifying dried egg white (DEW) with galactomanan (GM) through Maillard reaction can improve the gel properties and the water–holding capacity of such DEW (Matsudomi et al. 2002). The gel was firmer and more transparent at broader range of pH and the NaCl concentration of the medium according to these authors. Therefore, the combination of TGase treatment and the Maillard reaction with galactomannan may provide the synergism effect that helps further improve the pea protein extract functionalities to levels comparable to egg proteins.

5.2.2. Cookie sensory

Sensory was conducted on cookie using 50 panelists. Again, 4 attributes were evaluated including appearance, texture, flavor, and overall acceptability and the results were summarized in Table 26.

The result showed that there was no significant difference in appearance between samples which reflected the physical test results. The physical tests indicated the similarities in length and height of all samples with very small differences and this caused almost the same appearance scores in sensory evaluation of all three samples (Table 26).

Table 26. Sensory evaluation* of cookie attributes of different cookie formulas.

Formula***	Appearance**	Flavor**	Texture**	Overall acceptability**
Egg	7.11 ± 1.27^{a}	6.98 ± 1.43^{a}	6.71 ± 1.82^{a}	6.88 ± 1.57^{a}
PPI	7.01 ± 1.37^{a}	6.69 ± 1.58^{ab}	6.62 ± 1.71^{a}	6.73 ± 1.47^{ab}
TGase	7.00 ± 1.39^{a}	6.35 ± 1.55^{b}	5.95 ± 1.66^{b}	6.39 ± 1.44^{b}

* Values were calculated on a 9–point hedonic scale.
** Samples with different letters indicate significant differences ($p < 0.05$).
*** Egg: cookie made with egg, PPI: cookie made with pea protein isolate, TGase: cookie made with TGase–treated pea protein isolate.

The remaining attributes showed a different trend where the egg scored highest in all flavor, texture, and overall acceptability, followed by the PPI and the TGase. This was opposite with the cake result where egg cakes scored the lowest. In cookies, the batter was controlled in the way that gluten matrix was not formed and the shape of the cookies was determined mostly by the fat and sugar melting, thus the role of egg or egg replacers became less important as in case of the cake. This might explain why texture and overall acceptability were not significantly different between the egg and PPI samples and between the TGase and PPI samples. A significant difference between the egg and TGase samples in texture was because of slight stickiness of TGase cookies, which was not much accepted by consumers. Besides, TGase cookies also received a lower score in flavor, which was caused by off–taste and strange flavor as similarly found in case of TGase cakes. These two attributes caused a reduction in overall acceptability score of TGase and made it significantly different from the egg and the PPI samples. Flavor was a problem to TGase as "off–taste" was the most comments that TGase samples received. Again, solution for this could be an incorporation of a purifying step into the current extraction process which can help removing unwanted substances causing off–taste and off–flavor in baked products.

In general, sensory evaluation showed that the PPI and the TGase performed comparably. The PPI performed slightly better than the TGase in the real food applications, i.e. cakes and cookies, but such a difference was not enough to cause a significant difference between the two. The overall acceptability of the TGase samples was negatively affected by off–flavor and strange aftertaste in both food systems, resulting in lower scores compared to that of the PPI samples. However, the score could be improved if off–flavor and aftertaste problem was fixed. Besides, the TGase showed a better performance in terms of physical tests, especially in the cakes where the role of egg was very important. This suggested that the TGase could be better than the PPI in replacing egg in food systems if the off–flavor and aftertaste problem was corrected. Considering results from both physical test and sensory evaluation, both the TGase and PPI were incomparable to the egg, meaning that the TGase treatment alone was not sufficient to enhance the PPI functionalities to levels comparable to those of egg protein.

GENERAL SUMMARY AND CONCLUSION

PPIs have been introduced commercially but the application of PPI is still limited. Poor gelling property is one of the main barriers to its application in food systems.

The objectives of this research were to: 1) optimizing the extraction protocol based on alkaline extraction/isoelectric precipitation method for yellow pea to maximize yield while retaining protein nature; 2) modifying PPIs to improve gelling functionality by applying TGase treatment; 3) characterizing PPIs and TGase–treated PPIs extracted from optimized process; 4) Evaluating PPIs and modified PPIs as egg replacers using physical and sensory methods.

Important results and conclusions identified in this research were as following:

1) Extracting pH of 10.0, precipitating pH of 4.3, flour–to–water ratio of 1:6, and 30 minute extraction time were found to be optimum values for pea protein extraction. The spray drying method resulted in lower protein content, i.e. <60% vs. >88%, in final pea protein extracts but at least 6.2% higher protein yield compared to freeze–dry method. However, functionalities of pea protein products from both methods were comparable at final pH of 7.0.

2) TGase treatment at pH 6.0 provided the best overall functionality improvement in native PPIs compared to other pH levels. The SDS–PAGE results suggested that the large–molecular–weight proteins (~ 90,000 Da) and medium–weight–proteins (~50,000 – 80,000 Da) were main substrates for TGase catalyzed reaction whereas most of the proteins in the low–molecular–weight fraction (< 45,000 Da) were not involved in the reaction. RVA results indicated that TGase treatments at pH 6.0 and 7.0 resulted in

biggest viscosity improvements but the former showed a better stability and consistency. Functionality tests indicated that modified PPIs possessed a better viscosity profile than the native PPIs (controls) but no improvement in gelling capacity and only minor impact on foaming and emulsifying properties.

3) PPIs and modified PPIs extracted at optimal conditions had > 88% protein content and < 0.5% total starch which are comparable to literature values. PPIs had a very similar protein molecular weight profile as its original flour and showed better protein fractionation compared to the commercial PPI. Most of protein fractions in the control presented in molecular region from 45,000 Da to 66,200 Da. In contrast, TGase–treated PPIs had very few protein bands in all regions from 200,000 Da down to 21,000 Da. Most of their protein involved in TGase catalyzed reaction and formed large aggregates which had molecular weights larger than the gel pore size, thus cannot travel through the gel and retained in the wells. PPIs performance greatly depended on their final pH. The foaming capacity, foaming stability, and emulsion capacity were significantly improved when the final pH of PPIs was adjusted from 4.3 to 7.0.

4) Sensorial evaluation showed different behaviors where PPI and TGase–treated PPI had a higher overall acceptability compared to egg in cakes but not in cookies. Egg produced a better cake volume and texture but received a lower score in overall acceptability compared to PPI and TGase–treated PPI. In contrast, physical test of cookies showed no difference between cookies made from egg and from PPIs but the former received a better overall acceptability in sensorial test.

The hypothesis is that untreated pea protein isolates are poor in gelling capacity but this can be corrected by modifying pea protein isolates with suitable enzymes. With

an improvement in gelling capacity, modified pea protein isolates can be used to replace egg in cakes and cookies. The TGase was an enzyme used in this project to improve gelling capacity of pea protein isolates. Although, TGase treatment improved viscosity of pea protein slurries, it did not improve pea protein isolates' gelling capacity. Sensory evaluation showed different results but overall, indicated that TGase treatment alone was not sufficient to enhance the PPIs functionalities to levels comparable to those of egg proteins.

FUTURE WORKS

Future work is suggested in which the research should study a combination of TGase treatment with other treatments, e.g. Maillard reaction between PPIs and carbohydrates, to confirm if modified PPIs can fully replace egg in cakes and cookies. Other real food systems such as dressing, energy bars … should also be included to extent the scope and role of modified PPIs in food industry.

In terms of extraction process, current project was accomplished based on small–scale extractions which caused larger variations in final PPI quality and the extraction yield. Thus, a large–scale extraction should be considered in future works to improve yield and to gain the stability in quality tests. Purifying step should also be included in the extraction process in order to remove unwanted substances in final pea protein isolates that caused "strange" aftertaste in cakes and cookies.

Gelling properties are commonly tested using a rheometer which is currently unavailable in our department. This led to the difficulty in comparing this research result with literature values. Therefore, a rheometer is in need for future works.

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