CANOLA PROTEINS AND GLUCOSINOLATES: EFFECT OF PROTEIN ISOLATION AND POST-ISOLATION PROCESSING

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

Department of Food Science
University of Manitoba
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THE UNIVERSITY OF MANITOBA

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DEDICATION

To my family, for their love and continual encouragement during this challenging journey of my life, and for teaching me to be strong in any circumstances.

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ABSTRACT

Knowledge of the behavior of problematic factors, including the glucosinolates and their degradation products, is important if increased utilization of canola protein in human food is to be realized. Using a series of samples representing various stages in the protein micellar mass (PMM) process, the goals of this work were to determine how effective this process is in recovering protein and removing glucosinolates from low-temperature heated canola meal. Furthermore, the effects of post-isolation processing on the residual glucosinolates and glucose, and how these influence isolate color were examined.

In addition to the standard gas chromatography (GC) technique, the thiocyanate (SCN^-) ion determination and a diabetic test kit technique were used and evaluated as methods for assessing the glucosinolate levels in canola protein isolates. These latter two are based on the measurements of free SCN^- ions and the glucose released upon the glucosinolate decomposition, respectively. Our results showed a good agreement (R^2 = 0.93) between the GC method and the estimate of total glucosinolates from the diabetic test kit technique although an overestimation of glucose was observed possibly due to the presence of free glucose from other sources in the canola samples.

Ultrafiltration was an important stage in recovering canola protein, as well as reducing the glucosinolates. The Vivaflow 200 tangential ultrafiltration system was significantly better than the Amicon stirred cell system in achieving both functions, although removal of glucosinolate was not as great. Poor protein recovery (<12%) from canola meal has suggested that the PMM process is a selective technique for isolating only 12S salt-soluble globulins.

Processing conditions involving alkaline, high heat and low salt concentration significantly promoted the indole glucosinolates to decompose and release free SCN^- ions. Results on glucose level of protein isolates suggest a mixture of monosaccharides, including glucose, was readily formed when exposed to alkaline pH at low temperatures whereas the conversion from mono- to disaccharides took place in an acidic environment. A significant inverse relationship between lightness (Hunter color L* values) and glucose content, and a direct relationship between yellowness (b* values) and glucose content in the isolate could be traced to an undesirable Maillard reaction. The prevention of the glucosinolate decomposition and darker color caused by the Maillard reaction can be obtained by keeping the pH and temperature low.

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1.0 INTRODUCTION

Currently food scientists and the food industry have recognized canola as a potential source of food-grade plant protein. However, problem of underutilizing canola protein from the large volume of canola meal by-product must be overcome so that the protein can be consumed by humans thus increasing the profitability of the oilseed in food, and any other related industries. Despite the fact that plant breeders have successfully reduced the total aliphatic glucosinolates to no more than 30 μmole per gram of canola meal in canola varieties (Eskin and McDonald, 2000), glucosinolates are still one of the problematic factors present in canola. They are considered as antinutritional factors since some of their degraded products have significant goitrogenic and toxic effects in animals (Mithen *et al.*, 2000). Thus, the use of canola protein in humans has been prevented by these undesirable antinutritional factors. One protein isolation method, known as the protein micellar mass (PMM) process, has been developed to produce food-grade high quality canola protein. Employing only canola meal, salt and water, the PMM isolation method is claimed to be a mild and selective technique to isolate native protein with low levels of antinutritional factors (Burgess, 1991).

The three main stages of the PMM procedures, extraction, ultrafiltration, and dilution and precipitation, were examined to understand mass balance of protein and glucosinolates in the isolation process. NaCl, claimed as the most frequently used solubilizing agent (Burgess, 1991; Ismond and Welsh, 1992; Léger and Arntfield, 1993; and Murray, 2001), was used to extract canola protein. Following the extraction stage, the salt-extracted canola protein was concentrated and purified by ultrafiltration.

Inclusion of an ultrafiltration system into the PMM procedure has proven to be efficient in removing glucosinolates with minimal loss of proteins (Tzeng *et al.*, 1990a). Two types of ultrafiltraition systems, including stirred ultrafiltration cell and tangential flow ultrafiltration, were compared. By reducing the ionic strength of precipitation environment with the optimum dilution factor of one to six (Burgess, 1991), precipitation of the purified salt-extracted canola protein was the final stage of the PMM procedure. It is hoped that the PMM isolation process can result in higher canola protein recovery with minimal recovery of glucosinolates. Therefore, the first objective of this work was to demonstrate a mass balance on canola protein and glucosinolates during the PMM procedure. Evaluation of protein and glucosinolates as value-determining compounds are of particularly significance. This study also focused on qualification and quantification of total and individual glucosinolates, including the aliphatic and aromatic glucosinolates.

Glucosinolates in canola products can be determined through direct determination or indirect measurements of glucosinolate degradation products (Szmigielska *et al.*, 2000). Gas chromatography (GC) was selected as the direct method to determine the nature and levels of individual glucosinolates in the canola samples. The indirect methods measure the enzymatically or chemically released products, such as thiocyanate, isothiocyanate, nitrile, sulfate, and glucose, to estimate the glucosinolate levels (Szmigielska *et al.*, 2000). Applications of new methodologies such as using diabetic test kits are currently being evaluated to estimate the total glucosinolate level in canola (Vescio *et al.*, 2001). Accordingly, part of the objectives in this work was to evaluate the use of a diabetic test kit, as well as the thiocyanate (SCN^-) ion determination used by Campbell and Slominski (1990) to assess the extent of thermal

degradation of indole glucosinolates in canola seed and meal, as methods for monitoring the release of glucose and glucosinolate degradation products, respectively, by comparing to the standard GC technique.

Attempts have been made to apply a number of processing treatments, such as roasting, cooking and fermentation (Mahajan *et al.*, 1999), as a means to enhance the value of protein isolates in food systems in order to increase the acceptability of these proteins. However, the quality of protein isolates, including their physical and chemical attributes, may change upon the influence of processing conditions. Processing treatments can lead to great variation in glucosinolate degradation (Campbell and Cansfield, 1983; Sørensen, 1990), and degradation products are believed to exert a greater variety of antinutritional and toxic effects than the intact glucosinolates (Sørensen, 1990). Chemical changes of soluble sugars are another important chemical attribute of protein isolates to be evaluated upon processing as their presence is also claimed to affect the quality of plant proteins. Similar to glucosinolates and sugars, color, an important aspect of food acceptance, of protein isolates may change when subjected to processing treatments. As a result, another objective of this work was to examine the effects of processing factors on glucosinolate decomposition, change of sugar levels and color of canola protein isolates. Different pH, heat and salt concentration were used to demonstrate the behavior of canola glucosinolates and glucose in isolates, as well as isolate color, in potential food systems. In all, knowledge of protein and glucosinolates as value-determining components throughout the PMM procedures, and the possible glucosinolate decomposition and change of isolate color upon different processing conditions are important in order to achieve an optimum production of food-grade high quality canola protein isolates.

2.0 LITERACTURE REVIEW

2.1 Canola and Its Proteins

Canola, known in many countries as rapeseed, refers to cultivars of an oilseed crop that has a long history of use in Europe and Asia (Shahidi, 1990). As it is a cool season crop, canola has become one of the most valuable agricultural commodities in countries such as Canada, China and some European countries. Canada is the leading exporter of canola seed in the world, exporting an average volume of 3.4 million tons annually over the period 1997-2003, but is involved in less than 10% of the value-added processing in the world (Canola Council of Canada, 2003). Therefore, canola remains a potential source in Canada's food industry for value-added processing.

Canola is conventionally processed into oil and defatted canola meal by employing an extraction process. Oil is the main product where it is used as vegetable oil or a functional ingredient for the food industry. However, the meal, a major by-product that is used primarily for animal feed, plays an important role in determining the economic value of canola. Thus, the application and value of defatted canola meal has been considered for many years.

Interest in processing canola proteins for human consumption has increased due to the fact that production volume of the defatted canola meal is large and it contains a large amount of protein. The protein content of canola meal varies with variety and environment, but the average protein content is 40% (Shahidi, 1990). In addition, a continuing shift in consumer preference to plant protein over protein from animal sources has increased the potential commercial value of canola protein. Therefore, it

would seem that there is huge potential for application of this valuable source of protein in food.

2.1.1. Protein Isolation

Canola protein, more specify the 12S globulin, was first isolated by Bhatty and coworkers (1968) by extracting the defatted rapeseed meal with a NaCl solution, followed by a dialysis-precipitation against water and finally purifying the protein with chromatographic separation. Due to the fact that the 12S globulin is the major storage protein in canola seed, study of the isolation and subsequent characterization of canola proteins has focused primarily on the 12S globulin. Subsequently, numerous processes for isolating the 12S globulins have been developed and widely documented (Naczk and Shahidi, 1989; Tzeng *et al.*, 1990b; Chen and Rohani, 1992; Ismond and Welsh, 1992; Xu and Diosady, 1994; Klockeman *et al.*, 1997; Murray, 2001; Xu and Diosady, 2002). One of the current methodologies is the protein micellar mass (PMM) method that employs only canola meal, table salt and water. It is claimed to be a mild and selective technique to isolate native protein with low levels of antinutritional factors (Burgess, 1991). It should be noted that work in this laboratory demonstrated the use of PMM method to isolate canola protein, and is, therefore, be the main isolation method discussed in this chapter.

2.1.1.1 Protein Micelle Phenomena

Although the PMM process appears simple, the related chemical interactions are complex (Murray, 2001). In 1981, Murray and his co-workers demonstrated the

formation of a viscous gelatinous mass of protein called PMM from fababean. They attributed the formation of these mass to non-covalent interactions between the constituent proteins. This approach involved the extraction of the protein in a high salt environment followed by the formation of insoluble PMM through a rapid reduction of the ionic strength of their surrounding environment. As proteins are amphiphilic molecules, they would self-associate to form microscopically large protein spheres, known as micelles, and hence the name was used. This approach has since been applied to the isolation of canola proteins (Burgess, 1991; Ismond and Welsh, 1992).

In a polar aqueous environment, hydrophobic interactions, which are non-covalent protein-protein interactions, play an important role in micelle formation. Hydrophobic interactions arise from an energetically unfavourable relationship between water and the non-polar residues of a protein (Nakai and Li-Chan, 1988). In order to reduce the entropic changes in the system, the protein is forced to fold into micelles with the non-polar residues coalesced together inside and the polar residue on the outside. Burgess (1991) has further concluded that the hydrophobic interactions are the non-covalent driving forces for the formation of these protein micelles.

Several studies have concluded that the formation of micelles is strongly influenced by the initial amount and type of protein residue, pH and ionic strength of the surrounding milieu, and the dilution factor used (Murray *et al.*, 1981; Ismond, 1984; Welsh, 1988; Burgess, 1991). In general, proteins with high numbers of hydrophobic amino acid residues have good micelle forming capacity. A comparison of several oilseeds proteins is shown in Table 1, where the 12S globulin from canola has the highest average hydrophobicity ($HØ_{ave}$) and non-polar side chain frequency (NPS), as well as the lowest polarity ratio (P). As a consequence, the 12S canola globulin is more

hydrophobic, and therefore has greater potential to form micelle compared to other oilseed proteins.

Table 1. Hydrophobicity values of various 12S plant globulin

Source of 12S plant globulin	Average hydrophobicity, $H\O_{ave}$ (cal/residue)	Non-polar side chain frequencies, NPS	Polarity ratio, P
Canola	1041	0.36	1.01
Sunflower	951	0.31	1.21
Soybean	944	0.30	1.35

Adapted from Schwenke *et al.*, 1981.

2.1.1.2 Protein Extraction, Recovery and Purification

As mentioned earlier, this discussion will focus on the PMM method of isolating canola protein, and therefore, special consideration will be given to the protein extraction using salt, and precipitation upon dilution, as well as recovery or purification process of the extracted protein. There have been a number of methods used to produce canola protein employing extreme treatments involving acids and alkalis. For example, Tzeng *et al.* (1990b), Chen and Rohani (1992), Xu and Diosady (1994), Klockeman *et al.* (1997), and Aluko and McIntosh (2001) had isolated canola protein by alkaline extraction. The choice of alkali has become a preferred route for number of researchers due to the fact that higher protein is yielded as higher solubility of canola protein is associated with higher pH values. Acidic precipitation has often been used to recover the extracted 12S globulin (Gillsberg and Turnell, 1976a; 1976b; Gillsberg, 1978). These acidic and alkaline chemicals may reduce the nutritional value and functional properties of the protein and also provide environmental challenges for clean-up and disposal

(Murray, 2001). Therefore, PMM method is an alternative route to produce high-quality canola protein in an environmental-friendly way.

A number of salt solutions have been used to extract protein by manipulating surface hydrophobicity of protein in micelle formation (Burgess, 1991). NaCl is a moderately stabilizing salt that produces a desirably strong micelle response (Burgess, 1991). Also, as it is an inexpensive, readily available and a recognized food ingredient, NaCl is used most frequently as a solubilizing agent in canola protein isolation (Burgess, 1991; Ismond and Welsh, 1992; Léger and Arntfield, 1993; and Murray, 2001). As a result, NaCl was selected as the milieu to extract canola protein for this study. However, Klockeman *et al.* (1997) have reported that only 51% of the canola protein was soluble in 5% w/v NaCl compared to 91% in 0.4% NaOH. This shows that canola protein is less soluble in salt solutions than in alkaline solutions.

Protein recovery has become an obstacle in achieving optimum production yield of canola protein isolates. According to Chen and Rohani (1992), the recovery of canola meal protein by precipitation at pH 3.7 averaged only 53%. Maximum precipitation has been reported to occur at a range of pH values including pH 4.0 (Aluko and McIntosh, 2001), pH 3.7 (Chen and Rohani, 1992) or pH 3.5 (Klockeman *et al.*, 1997). Alternately, while precipitating in this pH range, the addition of sodium hexameta phosphate (SHMP) (Chen and Rohani, 1992) or $CaCl_2$ (Tzeng *et al.*, 1990b) during protein isolation was found to improve the recovery of canola protein. As a result, it is hoped that the inclusion of the dilution technique for salt-extracted canola protein (Burgess, 1991; Ismond and Welsh, 1992; Léger and Arntfield, 1993; Murray, 2001) by reducing the ionic strength of precipitation environment can result in higher canola protein recovery.

According to Burgess (1991), extraction of the 12S globulin from defatted canola meal was optimal in 0.5 M NaCl at pH 6.0. Formation of the micelle did not occur at extreme pH conditions, pH 4 or 9, due to the strong repulsive forces created as a result of an increase in net surface charges. Following the extraction step, the amount of aqueous solvent used to dilute the high salt protein extract in order to bury the hydrophobic residues of the protein is also critical for micelle formation. As a result, Burgess (1991) also suggested that the micelle response is optimal at a dilution factor of 1:10 if the pH of the protein milieu was at pH 6. Thus, it has been concluded that hydrophobic interactions with a good balance of hydrophobic-hydrophilic force are critical for micelle formation.

After the protein is extracted, it is necessary to concentrate and purify canola protein prior to the precipitation step in order to allow the protein to interact and form micelles during precipitation (Ismond and Welsh, 1992; Murray, 2001). Ultrafiltration is an excellent purification process based on membranes with macromolecule-sized openings to allow permeation of water and small, low-molecular-weight dissolved components, while retaining large molecules (Tzeng *et al.*, 1990a). Therefore, it is an ideal process to concentrate the protein by separating low-molecular-weight contaminants. Incorporation of an ultrafiltration system into the protein isolation processes has proven to be effective in removing glucosinolates, with minimal loss of proteins (Tzeng *et al.*, 1990a). Ultrafiltration, in combination with a drying process of the supernatant following precipitation of canola proteins, has also been used to recover high levels of soluble protein representing the 2S canola protein in the supernatant (Tzeng *et al.*, 1990a; 1990b; Rubin *et al.*, 1990).

In addition to removing glucosinolates from canola protein isolates, there are other specific compounds that researchers try to reduce or eliminate as well. These include fiber, protease inhibitors, phytic acid and phenolics. Dehulling seems to be the most effective way to minimize excess fiber problem in most of the oilseed proteins. However, mechanical dehulling does not provide a good separation on small canola seeds (Niewiadomski, 1990) as the hull tends to adhere to the endosperm (Thakor *et al.*, 1995). Besides, dehulling is reported to adversely affect oil recovery (McCurdy, 1992), and, therefore, inclusion of the dehulling process is rarely applied in the canola seed industry. In most cases, protease inhibitors that cause reduced pancreatic activity and growth inhibition (Shahidi, 1997) are inactivated upon heating prior to consumption (Ceciliani *et al.*, 1994). However, Ceciliani *et al.* (1994) have reported that heating alone may not completely eliminate thermally stable protease inhibitors and, thus, their presence should be monitored during the preparation of any oilseed protein products. Phytic acid is capable of interfering with the availability of important divalent cations, such as zinc, iron, copper, and manganese, and as a result, high phytic acid content may decrease the bioavailability of essential minerals (Thompson, 1990). Early research has shown that minimal solubility of phytic acid is at the pH values of 10-12 (Gillsberg and Turnell, 1976a). Therefore, by extracting the protein from the canola meal at a pH value greater than 10, the formation of phytate, the salts of phytic acid, in the canola protein can be reduced due to the fact that the number of positively charged groups on the protein is decreased. However, despite the fact that PMM method isolates canola protein at a neutral pH, protein purification, such as ultrafiltration, seems to be the preferred route to reduce phytate levels in canola protein. Phenolic compounds in canola have become more of an obstacle in relation to their impacts on the flavour, color and mineral

availability in canola protein products (Shahidi, 1997). Not only effective in removing both intact glucosinolates and their breakdown products (Shahidi *et al.*, 1990a; Pecháček *et al.*, 2000), the use of a methanol-ammonia-water treatment has been also shown to be effective in significantly reducing the levels of phenolic compounds in oilseed protein isolates (Naczk and Shahidi, 1989; Wanasundara and Shahidi, 1994a; 1994b). More recently, Xu and Diosady (2002) has successfully used a treatment with Na_2SO_4 and polyvinyl pyrolidone (PVP) to decrease the levels of phenolic compounds to produce high-quality canola protein isolates. In all, it should be noted, however, that the glucosinolates will be the only antinutritional or toxic compounds to be addressed more in details and as they will be evaluated in relation to the quality of canola protein in this study.

The PMM method has been used to isolate canola protein experimentally for years (Burgess, 1991; Ismond and Welsh, 1992; Han, 1994). Efforts to modify the PMM method are still being undertaken to maximize the recovery of protein from canola while efficiently removing the undesirable compounds, including phytic acid, phenolic compounds and glucosinolates. While many researchers recognize the nutritional quality of canola protein, the potentially toxic or antinutritional factors must be addressed.

2.1.2 The Canola Protein

The canola protein consists of mainly salt-soluble globulins and water-soluble albumins (Norton, 1989). The albumins are the main metabolically active proteins. Representing 20% of total protein in the seed, they are responsible for the biosynthesis and degradation of globulins. On the other hand, as mentioned before, the globulins,