CHARACTERIZATION AND FUNCTIONAL PROPERTIES OF CANOLA PROTEIN HYDROLYSATES

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Angela Hooi Leng Han

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BY

ANGELA HOOI LENG HAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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ABSTRACT

Functional properties of proteins can be modified by the use of hydrolysis. Canola proteins (*Brassica napus*) prepared by the protein micellar mass (PMM) method were subsequently hydrolysed by acid, trypsin, chymotrypsin, bacterial and fungal proteases at varying time intervals. Different hydrolysis procedures showed different impacts on the molecular weight distribution, subunit analysis, isoelectric points and protein conformation as well as the surface hydrophobicity. Enzyme treatments cleaved PMM into smaller fragments compared to both acid methods. Some protein aggregation was observed in alternate acid hydrolysates. After hydrolysis with four different enzymes, the resultant hydrolysates showed isoelectric points in the range of 6.2-6.7 whereas all acid hydrolysates from both acid methods had isoelectric points in the range of 4.4-5.7. Fungal and bacterial hydrolysis methods provided the mildest conditions in terms of the enthalpy of denaturation and the thermal denaturation temperature. Overall, T5 had a greater aromatic surface hydrophobicity whereas C20 showed an increased aliphatic surface hydrophobicity. Nitrogen solubility, water holding capacity, fat absorption capacity, emulsion stability, foaming capacity as well as foaming stability of all the hydrolysates were investigated. Hydrolysates treated with chymotrypsin for 20 min (C20) showed the highest nitrogen solubility at pH 4.5 whereas acid hydrolysates of 4 h exhibited the highest solubility at pH 7.0. Hydrolysates prepared by acid hydrolysis for 7 h showed the best water holding capacity whereas chymotrypsin hydrolysates (10 min) exhibited the best fat absorption capacity. Emulsion stability was slightly decreased

from the original PMM in most enzyme hydrolysates and mild acid hydrolysates whereas this stability was significantly improved using an alternate acid hydrolysis method. An acid hydrolysate (A5) possessed the best foaming capacity whereas the A7 acid hydrolysate showed the best foaming stability. Relationships between molecular characteristics and functional parameters were established.

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DEDICATION

This thesis is specially dedicated to my beloved parents -
Tan Joo Kian and Han Jin Fong,
my brothers and sister -
Koon Juan, Lee Juan, and Michelle,
and my dearest husband,
Mickey Ma.

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I. INTRODUCTION

Tailoring the functional properties of proteins for meeting the complex needs of the manufactured food products can determine the effective utilization of protein in food systems. The use of plant proteins in food products is limited mainly due to the lack of desirable functional performance of these proteins in foods. Therefore, proteins usually require modification using enzymatic or chemical methods to improve such functional properties as solubility, whippability and emulsification activity. Generally, protein modifications for food utilization can be classified into three areas: (1) nutrition - introduction of deficient nutritional components into the protein or improvement in its digestibility by the biological agents; (2) functionality - alternations in the nonnutritional, but still useful, properties of the proteins (solubility, water or fat uptake, viscosity, etc.); (3) organoleptic properties - modifications in the taste and flavour of the protein material for improved palatability.

The main objective of this study is to obtain a canola protein hydrolysate which has optimal functional properties. Canola proteins were isolated using the protein micellar mass (PMM) procedure. The resultant PMM was subsequently modified using various enzymatic and chemical methods in order to obtain different hydrolysates. Following this, several molecular characteristics (including molecular weight, subunit analysis, isoelectric point, thermal stability, hydrophobicity) of the PMM and all hydrolysates were studied. Several functional properties (such as nitrogen solubility, water and fat binding, emulsion and foaming properties) were examined with the PMM

and all the hydrolysates in order to evaluate the possible use of canola protein hydrolysates in food systems. Then, the relationships of molecular characteristics of the proteins and their functional properties were considered so that some understanding of the relationships between the structure and function of proteins could be established.

II. LITERATURE REVIEW

A. Canola Protein - General Considerations

1. Protein Content and Protein in Canola Seed

Canola is a major oilseed crop in Canada. Knowledge of the composition and properties of the components of canola is important for achieving an optimal production of protein flours, concentrates and isolates (Mieth *et al.*, 1983). In terms of content, canola ranges from 11-42% protein; this is influenced by genetic and environmental factors (Mieth *et al.*, 1983).

Canola meal contains three protein fractions : salt-soluble globulins or storage proteins, water-soluble albumins and alkali soluble proteins (Norton, 1989). These fractions can be separated not only by ultracentrifugal, chromatographical and electrophoretical behaviour, but also by differences in isoelectric points as well as solubilities (Mieth *et al.*, 1983). According to Norton (1989), the albumins represent the majority of the metabolically active proteins which are responsible for the biosynthesis and degradation of globulins. Albumins are located in the cellular cytoplasm in the seeds. Globulins, which serve as nitrogen reserves for the embryonic axis during germination, constitute the majority of the storage proteins. Storage globulins are situated in the protein bodies in the parenchyma cells of the seeds (Norton, 1989).

Various protein fractions are commonly designated by their sedimentation coefficients. In general, *Brassica* spp. possess four protein fractions, namely 1.7S, 7S, 12S and 15S or 17S (Bhatty *et al.*, 1968; MacKenzie and Blakely, 1972). According to

Norton (1989), the 12S globulin (cruciferin) and the 1.7S albumin (napin), which account for about 60% and 20% of the total seed proteins respectively, are two of the major seed proteins. The 12S globulin is a high molecular weight, neutral complex, composed of several polypeptide chains. In contrast, the 1.7S albumin is a low molecular weight, basic protein, composed of two disulfide-linked polypeptide chains (Ericson *et al.*, 1986). Norton (1989) stated that the 7S protein is less widely distributed in *Brassica* spp. Prakash and Rao (1986) concluded that the 15 S or 17 S fraction is a polymer resulting from possible aggregation of the 1.7S, 7S, or 12S proteins rather than being inherently present in the seed.

2. Physico-chemical Properties of the Canola 12S Globulins

a. Molecular Weight and Subunit Profile. The 12S globulin represents an oligomeric protein with a molecular weight of 300,000 (Schwenke *et al.*, 1983). As mentioned, the 12S globulin is the major storage protein in the seeds of *Brassica* spp. According to Bhatty *et al.* (1968), 21-33% of the nitrogen in NaCl extracts of defatted rapeseed varieties or 18-28% of the total seed nitrogen correspond to this protein.

The 12S globulin was first isolated by Bhatty *et al.* (1968) from oil-free rapeseed meal by extraction with 10% NaCl, precipitation by dialysis against water, and chromatographic separation on Sephadex G-100. Other techniques such as the application of a combined gel and ion-exchange chromatographic purification method have been used by Schwenke and co-workers (1981) to isolate the 12S fraction. The physico-chemical properties and structure of the 12S globulin are summarized in Table

TABLE 1. Physico-chemical properties and structures of the 12S globulin from rapeseed[1]

PROPERTY	VALUE
Molar Mass (g/mol)	300,000
Isoelectric Point	7.2
Sedimentation Coefficient $S^{o}_{20,w}$ [10^{-13} sec]	12.7
Diffusion Coefficient $D^{o}_{20,w}$ [10^{-7} m^2/sec]	3.8
Stokes Radius (Rs, nm)	
Quasielastic light scattering	5.7
Gel chromatography	5.5
Partial Specific Volume (ml/g)	0.729
Frictional Ratio (f/fo)	1.28
Molecular Weight	
Sedimentation velocity and diffusion	300,000
Sedimentation velocity and gel chromatography	294,000
Dimension (nm)	
Electron microscopy	11.3 x 11.3 x 9.2
Small angle scattering	10.5 x 10.5 x 9.2

TABLE 1. (Cont'd)

PROPERTY	VALUE
Secondary Structure	
α-helix	11%
β-sheet	31%
aperiodic	58%
Quarternary Structure	trigonal antiprism
number of subunits	6
number of polypeptide chains	12
Molar Mass of Polypeptide Chains (g/mol)	
PPC1	18500 $\pm$ 800
PPC2	21100 $\pm$ 500
PPC3	26800 $\pm$ 900
PPC4	31200 $\pm$ 1600

[1] adapted from Mieth *et al.* (1983)
Schwenke *et al.* (1983)
Prakash and Rao (1986)

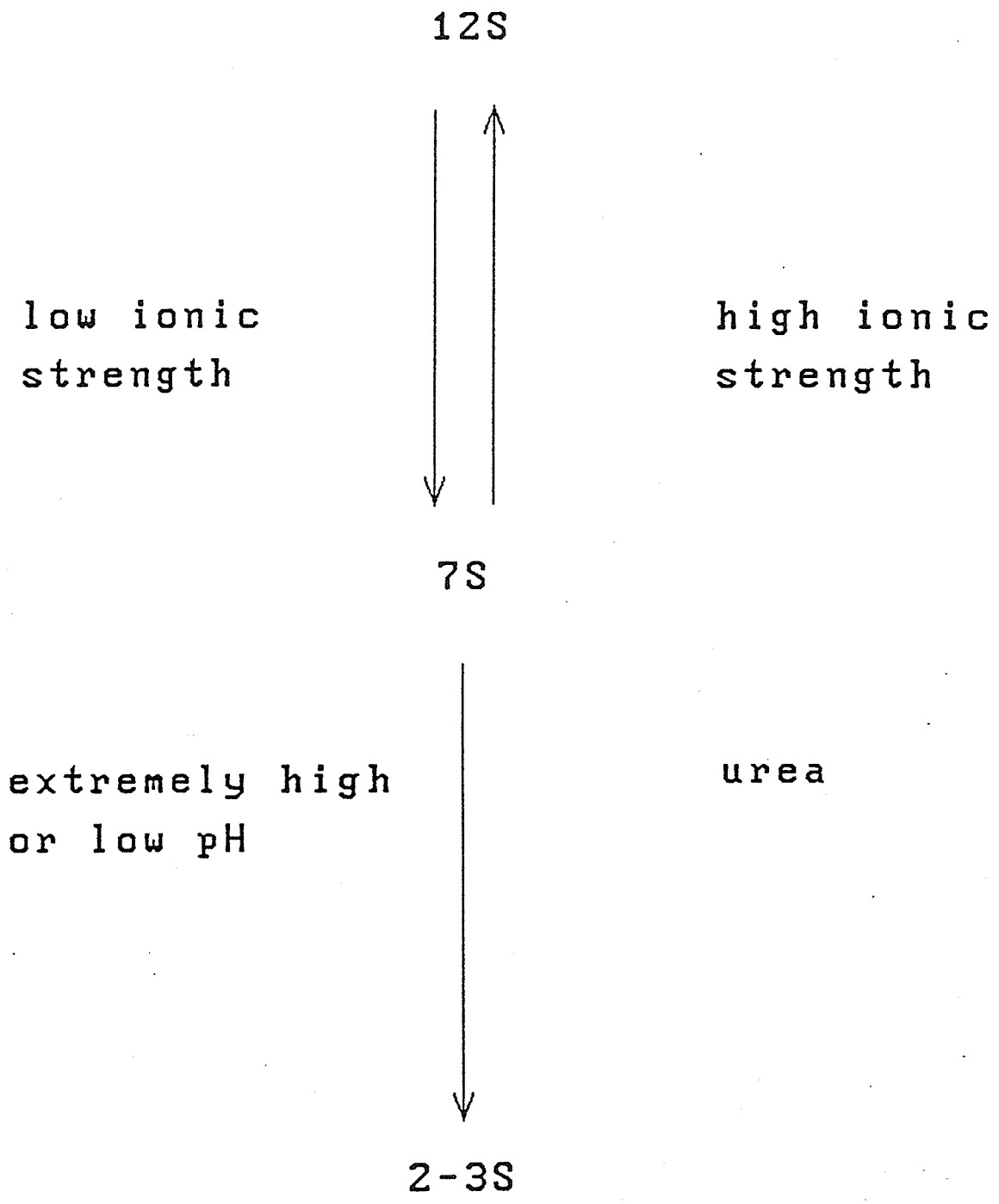
1.

Reichelt *et al.* (1980) has used electron microscopy to study the quaternary structure of the 12S globulin and they have proposed that the subunits are arranged in the form of a trigonal antiprism with a point symmetry of 32. Schwenke *et al.* (1983) also confirmed that the quaternary structure was composed of six ordered subunits arranged as a trigonal antiprism with each subunit made up of two polypeptide chains. These polypeptide chains have molecular weights in the range of 18,500 to 31,000 (Table 1).

b. Dissociation Profile. The 12S globulin has been shown to dissociate in the presence of urea and under varying pH conditions plus ionic strengths (MacKenzie, 1975). Fig. 1 illustrates the dissociation profile of the 12S globulin.

Bhatty *et al.* (1968) showed that the rapeseed protein exists as an hexameric native 12S globulin in high ionic strength solution. Schwenke *et al.* (1983) concluded that the 12S globulin dissociates as a trimeric 7S unit in a low ionic strength solution. However, this 7S unit will re-associate nearly completely when placed in high ionic strength solution. Contrary to this reversible dissociation, Goding *et al.* (1970) proved that the 12S globulin undergoes an irreversible dissociation into 2-3S monomeric subunits in the presence of strong dissociating agents (4-6M urea), especially in an acidic condition of pH $\leq$ 3.6.

FIGURE 1. The association - dissociation profile of the 12S canola globulin. (Schwenke *et al.*, 1981; 1983)


12S
low ionic strength
high ionic strength
7S
extremely high or low pH
urea
2-3S

B. Micelle Phenomena

When amphiphilic molecules are dissolved in water they can achieve segregation of their hydrophobic portions from the solvent by self-aggregation (Tanford, 1973). The aggregated products are known as micelles. In 1981, Murray *et al.* used a noncovalent approach to processing and utilizing plant proteins such as fababean protein, by formation of a viscous gelatinous mass called a "protein micellar mass" (PMM). The process involved the solubilization of the protein in a high salt environment followed by a rapid reduction of ionic strength and hence the formation of insoluble protein micelles.

1. Molecular Forces for Micelle Formation

Protein-protein interactions may occur as a result of either covalent or noncovalent interactions. According to Karp (1984), covalent interactions usually refer to high energy disulfide linkages. Murray *et al.* (1981) and Cheftel *et al.* (1985) emphasized the role of the disulfide bond as more of a stabilizing than a conformation directing force in micelle formation. Therefore, noncovalent forces may be of prime importance in micelle formation (Murray *et al.*, 1981). These noncovalent forces include van der Waal forces, hydrogen bonds, electrostatic interactions as well as hydrophobic interactions.

However, Burgess (1991) concluded that van der Waals forces, hydrogen bonds and electrostatic interactions play no or little role in micelle formation and association. Hence, hydrophobic interactions are thought to be the entropic driving force for micelle formation (Burgess, 1991). According to Nakai and Li-Chan (1988), the hydrophobic effect arises when water interacts with the nonpolar residues of a protein, and this

interaction frequently decreases its entropy. In order to minimize the unfavourable entropy changes, Nakai and Li-Chan (1988) suggested that the contact surface of protein with water should be reduced by forcing the nonpolar portion to coalesce together into droplets or globules. Therefore, the protein chain is forced to fold into a micellar structure with the hydrocarbon moiety on the inside of the globule and the polar groups on the outside. Thus, hydrophobic interactions are important in the formation of protein micelles.

2. Criteria for Micelle Formation

Several criteria are important for micelle formation. Reynolds (1979) proposed that protein molecules will self-associate and form micelles at a critical micelle concentration (cmc). Furthermore, the amount and type (aliphatic or aromatic) of hydrophobic residues are critical for micelle formation (Tanford, 1973). Bigelow (1967) concluded that the knowledge of amino acid composition and hydrophobicity of the protein is important to identify proteins with good micelle forming capacity.

In 1984, Ismond proposed that proteins with high numbers of hydrophobic amino acid residues generally have a potential for micelle formation. Furthermore, the protein molecule must possess sufficient polar residues on the surface to bury the hydrophobic portion inside the moeity. Nakai and Li-Chan (1988) stated that some hydrophobic residues were able to be positioned on the outside as well as inside the protein; therefore, the importance of this flexibility and balance of internal and external hydrophobic groups will become more apparent when considering the effects of environmental manipulation

on micelle formation.

The amount of aqueous solvent used to bury the hydrophobic groups is also important. The type of micelle response is strongly dependent upon the dilution factor (Burgess, 1991).

3. Isolation of Protein Using Micelles

As mentioned before, Murray *et al.* (1981) have isolated seed globular proteins using a protein micellar mass (PMM) procedure. This involved stirring defatted meal in a high ionic strength salt solution and then diluting the high salt protein extract (HSPE) containing the solubilized protein, with cold distilled water. The low water solubility of the globular protein plus the decrease in ionic strength resulted in protein aggregation, micelle formation and interaction, and precipitation of the protein. In 1985, Arntfield *et al.* recovered 42.5% of the protein from fababean whereas Welsh (1988) recovered 4% protein from canola. Burgess (1991) modified the procedure used by Welsh and was able to recover approximately 31% protein from canola. The two important criteria in this procedure are the extraction step and the dilution of the high salt protein extract (Burgess, 1991).

Burgess (1991) found that extraction of canola proteins in 0.5M NaCl at pH 6.0 was optimal. Micelle formation did not occur in extreme pH environments (pH 4 or 9) due to the strong repulsive forces. However, the dilution factor was strongly dependent on the pH and ionic strength of the extracting environment. In this case, a dilution factor of one to six was used to obtain the optimal micelle response.

In 1992, Ismond and Welsh also used the PMM procedure to isolate the canola globular protein. They proposed that 0.1M NaCl/0.1M NaH_2PO_4 buffer at pH 5.5 was the best condition to remove both phytic acid and phenolic compounds whereas 0.01M NaCl/0.01M NaH_2PO_4 buffer at pH 5.5 was the optimal condition to remove glucosinolates.

4. Effects of Environmental Manipulation on Micelle Formation

Research by Murray *et al.* (1981), Ismond (1984), Welsh (1988) and Burgess (1991) suggested that the formation and association of micelles are strongly affected by the initial protein concentration, pH and ionic strength of the solubilizing environment as well as the dilution factor.

In general, the micelle response at any protein concentration depended on the surrounding milieu. The surface properties of the protein can be altered by changing the solubilizing environment. As previously mentioned, hydrophobic interactions with a good hydrophobic-hydrophilic balance as well as slight repulsive electrostatic forces were critical for micelle formation and interaction (Burgess, 1991).

Ismond *et al.* (1986a) identified a strong micelle response from fababean vicilin occurring at pH 6.0 to 6.8. Further micellization did not occur above the optimal pH range due to the changes in protein conformation as a result of the increase in net negative surface charge. Furthermore, a decrease in surface hydrophobicity, indicating that a reduction of exposed nonpolar residues occurred at higher pH values, resulted in fewer micelle interactions.

Ismond *et al.* (1986b) selected various salts to study the influence of hydrophobic interactions on micelle formation. Ismond *et al.* (1986b) concluded that nonchaotropic salts were inadequate to promote extensive hydrophobic associations between micelles as the nonpolar residues were buried within the protein molecule. Moderately stabilizing salts ($NaC_3H_6O_7$, NaBr and NaCl) were shown by Ismond *et al.* (1986b) and Georgiou (1987) to be the best environments to produce highly interactive networks from fababean micelles ($\mu \leq 1.0$). In these situations, the hydrophobic-hydrophilic forces were balanced.

C. Hydrolysis of Proteins

1. Hydrolysis as a Tool to Modify Proteins

Many food proteins, particularly those from plant sources, require modification to improve such functional properties as solubility, emulsification and others (Shih, 1992). Modification of protein functionality can also make food products better suited for human nutritional utilization, therefore increasing the world's food supply (Hamada, 1992). As a result, Hamada (1992) concluded that the purpose of protein modification was to create new and unique products that would possess better functional properties in food systems than the unmodified protein.

Chemical and enzymatic hydrolysis are the two most popular forms of protein modification. The final product, referred to as a protein hydrolysate, is defined as a mixture containing amino acids and other substances such as salt and peptides, obtained by the hydrolysis of plant or animal proteins (Olsman, 1979).