Purification, Characterization and the Micelle Response of the 12S Canola Globulin.

by

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A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

Food Science Department

June 1991

ISBN 0-315-76670-0

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ACKNOWLEDGEMENTS

The successful completion of this thesis was enhanced by the invaluable advice and assistance of several people. Dr. Susan Arntfield was instrumental in giving my thesis direction and meaning as well provided moral support, guidance, patience and friendship. An on going inspiration and a key player in my masters program was Dr. E.D. Murray. Dr. M.A.H. Ismond was instrumental in establishing the groundwork of my research and financial support in coordination with the Food Science department. Technical assistance, support and friendship was generously provided by Aniko Bernatsky, Malgorzata Szcygiel, Janine Grabowecky, Jim Rogers and Paul Stephen. I have developed great admiration for these individuals and their contributions will always be fondly remembered.

I would like to thank Dr. Joe Mazza of Agriculture Canada for his guidance and interest in initiating the continuation of my formal education. The assistance and personal communications with Dr.LeJohn and Dave Thiessen were also valuable for the successful completion of my thesis.

I have had the opportunity to make a number friends which helped make this time memorable, exciting and fun filled. In addition, my family has been an endless source of inspiration and energy with their unquestioning support, and guidance. Without the guidance, patience and friendship of my biggest supported, Terry, I would never been successful.

ABSTRACT

Various steps in the PMM procedure applied to canola meal were examined to optimize protein recovery. In the extraction step, solubilization was achieved with 0.5M NaCl. Using chromatographic and electrophoretic techniques, the predominant extracted protein identified was the 12S globulin under all salt conditions; there was no evidence to indicate the presence of dissociated 7S form.

The impact of pH, NaCl concentration, protein concentration and dilution on protein conformation as well as the formation and interaction of protein micelles were evaluated using gel filtration chromatography, differential scanning calorimetry (DSC) and light microscopy. Variations in pH from 6 to 9 and salt concentrations between 0.1M and 0.5M resulted in no major conformational changes (similar enthalpies of denaturation) but higher pH values and salt concentrations were associated with increased denaturation temperatures reflecting increased molecular stability. At pH 4, however, the 12S globulin dissociated into its 2-3S. This dissociation inhibited the formation of protein micelles.

To efficiently isolate proteins with the micellar mass procedure, it is desirable to produce highly interactive micelles while keeping protein concentration and dilution ratio to a minimum. Such reactions were obtained at pH 6, with NaCl concentrations of 0.3M and 0.5M, protein concentrations as low as 25mg/mL and a dilution ratio of at least 1:10.

Higher dilution ratios and protein concentrations resulted in a more intense reaction. Lower NaCl concentrations resulted in precipitation prior to dilution and reduced interactions between micelles upon dilution. Variations in pH also effected the formation of micelle networks. At pH values away from the isoelectric point (e.g. pH 4, 8 and 9), the increased net charge was detrimental to good micelle interaction in addition high dilution ratios further reduced the micelle rating. Near the isoelectric point (pH 7), the tendency for pre-dilution precipitation was enhanced and the micelle response was more dependent on salt concentration. Networks formed at pH 7 tended to be delicate and required higher protein concentrations and dilution factors than at pH 6.

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INTRODUCTION

The canola industry has primarily devoted its attention to edible oil production. As a result, the full impact of canola as a human food source has not been fully realized. The residual meal produced during oil extraction contains 36-40% protein with a well balanced amino acid profile (Downey and Röbbelen, 1989). This high nutritional value has been well documented in the literature (Lo and Hill 1971a; 1971b, Girault, 1973) despite the presence of the antinutritional factors: glucosinolates, phenolics and phytic acid. The elimination of these antinutritional factors can be primarily accomplished by employing appropriate processing technology. To increase the utilization potential of canola meal, a variety of extraction conditions have been used to isolate canola proteins. In general, the drawbacks of these isolation procedures include low protein recoveries, poor functionality and high residual levels of antinutritional factors. For example, harsh extraction conditions, traditionally used with acidic isoelectric precipitation of alkali extracts, result in high yields with limited protein functionality (Sosulski and Bakal, 1969; Gillberg and Törnell, 1976a; Mieth *et al.*, 1983b; Cheftel *et al.*, 1985). This lack of functionality makes this methodology undesirable from a food production viewpoint. Thus, new methodologies were needed to maintain high protein yields and protein functionality, and to decrease antinutritional factors.

In attempts to isolate functional storage proteins, Welsh (1988) used the modified protein micellar mass (PMM) process developed by Murray

et al. (1981). The PMM isolate, containing 80% protein, was light in color and possessed only minimal amounts of problematic antinutritional contaminants. Concerns of structural integrity and protein recoveries, however, limited the implementation of this modified PMM methodology. By adapting this novel process to the physicochemical properties of the 12S canola globulin these concerns should be rectified. Chemically, the ubiquitous nature of this 12S canola globulin, cruciferin, makes the surrounding environment a key parameter in determining the molecular integrity of the protein. The 12S globulin reversibly dissociates to the 7S trimeric half molecule in low ionic strengths or irreversibly to the 2-3S subunit in pH extremes or the presence of denaturing agents (Schwenke *et al* 1981; 1983). In addition, reducing agents further cleave the 2-3S subunit to basic and acidic components. As a result, any extraction conditions promoting dissociation should be avoided. Furthermore, molecular weight detection techniques including chromatography, electrophoresis, ultrafiltration and differential scanning calorimetry should be utilized to ensure homogeneity and nativity of the 12S globulin system.

The modified PMM procedure utilized by Welsh (1988) could be further adapted to the physicochemical properties of the 12S canola globulin upon attaining a thorough understanding of the noncovalent forces responsible for enhanced micelle formation and self association. As a result, this knowledge must be gained through manipulation of various environmental conditions where the micelle response can be assessed. To predictably manipulate the micelle response for fababeans, a variety of pH environments and electrolyte media were employed by Ismond *et al.* (1986a;

1986b; 1990). In these studies, it was recognized the micelle phenomenon was not an all or none response as micelles undergo dynamic interactions to produce amorphous and three dimensional networks and proteins sheets under the appropriate conditions. Through environmental manipulation, the response was shown to be dependent on a critical balance of noncovalent forces operating at several levels. Ismond *et al.* (1986a; 1986b; 1990) demonstrated that for fababeans these forces were optimal in the presence of moderately stabilizing anions ($C_2H_3O_2^-$, Br^-, Cl^-) and when the protein contained a slight charge repulsion. Accordingly, moderately stabilizing anions were expected to exert strong responses in canola systems. Consequently, NaCl was selected as the desired experimental milieu.

To better understand the factors critical to the micelle phenomenon, protein concentration, protein to water dilutions, environmental pH and ionic strengths, were manoeuvred. Optimizing this micellization phenomenon, could ultimately provide insight to increasing the effectiveness, efficiency and feasibility of the PMM procedure on canola proteins. Eventually, this process could be successfully applied to the food industry as a simple and mild method for extracting relatively pure and native canola proteins. With commercialization, the production and marketing of a well balanced protein food ingredient could improve the feasibility of the production which would make it more competitive with other plant proteins.

Maximizing the PMM process for canola systems is the ultimate goal; however, the object of this research was to optimize the nitrogen solubility and to understand the micelle phenomenon of the canola

globulin. To maximize protein extractability, the meal was solubilized in various NaCl concentrations and compared to SHMP extractions which have become increasingly popular in the literature. After an optimum environment was established, the extracted protein was isolated, purified and characterized. Then, its ability to form micelles under various environments was monitored. This research, based on a model system, also constitutes the first step towards optimizing globulin isolation and enhancing the understanding of how to effectively manipulate canola proteins in order to provide the structural frameworks required in many food systems.

II. LITERATURE REVIEW

A. General Considerations of Canola Protein

1. Protein Content in Canola Seed

The protein content of canola seeds ranges from 11 to 42% and is influenced by a number of factors including genetics and the environment (Mieth *et al.*, 1983a). For example, the abundance of rain or application of nitrogen fertilizers increases the overall protein content (Ohlson, 1985). As the seed matures, there is an increase in amino acids and in turn an increase in high molecular weight globulins or storage proteins along with a decrease in nonprotein components. Daun (1984) indicated a negative correlation exits between oil percentage in the seed and the protein content in the meal.

2. Protein Composition

The meal is composed of three major protein fractions: albumins, alkali-soluble proteins and globulins. These proteins can be separated by differences in molecular weight, isoelectric points, solubilities, as well as chromotographical, electrophoretical and ultracentrifugal behaviours (Mieth *et al.*, 1983a). Canola proteins consist primarily of water soluble albumins and salt soluble globulins or storage proteins. The properties of the globulin and albumin proteins are compared in Table 1.

In the seed, albumins, located in the cytosol of the cell, comprise the majority of the metabolically active proteins and are responsible for biosynthesis and degradation of globulins (Wright, 1987; Norton, 1989).

TABLE 1. Comparison of globulin and albumins in canola / rapeseed

Property	Globulin	Albumins
Secondary Structure		
α-helix	11%	46%
β-sheet	31%	11%
aperiodic	58%	43%
Sedimentation Coefficient	12S	1.7S
Molecular Weight	300,000-320,000	12,000-18,000
Isoelectric Point	4.0-8.0	9.5-13.0
Solubility	-pH away from pI -in low ionic strength solutions	-independent of pH in aqueous medium -in mediums of low ionic strength
Techniques to precipitate the protein	-adjust pH to pI -decrease ionic strength by dilution or dialysis -salt out -heat coagulation	-salt out -ethanol -heat in pI range -complex with polyanions -detergents

Adapted from Mieth *et al.* (1983a)
Schwenke *et al.* (1983)

The predominant storage proteins, globulins, are deposited in protein bodies or the aleurone which are abundant in the storage parenchymous cells of the seed (Appelqvist and Ohlson 1972; Stanley *et al.* 1976). Globulins are utilized as nitrogen reserves during germination to aid the growth of the embryo or cotyledons and the seedling (Wright, 1987; Norton, 1989).

The number of protein fractions in the total protein is generally recognized by the sedimentation velocity pattern. In fact, it has become common practise to designate the protein fractions by their sedimentation coefficient. As indicated by Bhatty *et al.* (1968) and MacKenzie and Blakely (1972) *Brassica* species generally possess four components with sedimentation velocity coefficients of 1.7S, 7S, 12S, and 15 or 17S. In Brassica species, especially rapeseed varieties, the 7S fraction is very low or nonexistent (Bhatty *et al.* 1968; MacKenzie and Blakely, 1972; and Prakash and Rao, 1986). Norton (1989) suggested the 12S globulin (cruciferin) accounts for about 60% of the total seed protein of *B. napus* while the 1.7S protein (napin) is approximately 20%. In rapeseed, the 15 or 17S fraction is generally regarded as a polymer resulting from possible aggregation of the 2S, 7S or 11S rather than being inherently present in the seed (Prakash and Rao, 1986).

B. Physicochemical Properties of the Rapeseed / Canola 12S Globulins

1. Molecular Weight and Dissociation Profile

For reasons previously stated, past research has focused primarily on the physicochemical properties and the structure of the predominant 12S globulin, an oligomeric protein with a molecular weight of 295,000-

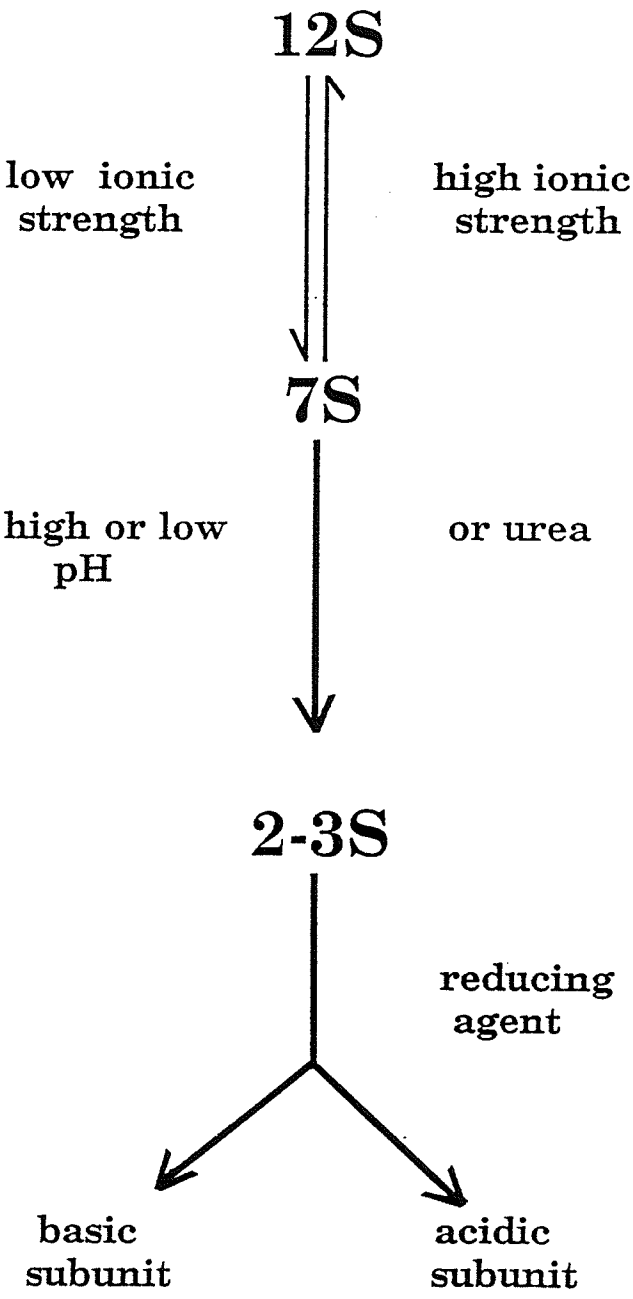
320,000. Schwenke *et al.* (1981) believed the previous discrepancies in the molecular weight determinations were due to the possible dissociation-association properties of the 12S canola globulin (Figure 1). Variations in solution composition, such as pH, ionic strength, and the nature and amount of salt present, could drive the reaction in the direction of the 7S trimer. Ionic strength appeared to be the main factor determining the form of the major component. In high ionic strength solutions the protein remained in a hexameric (12S) form. When the ionic strength was equal to or less than 0.5, the protein dissociated to the short lived trimeric, 7S form. Norton (1989) suggested the presence of the 7S protein was very limited and short-lived in *Brassica* species. The 7S proteins were thought to irreversibly dissociate to its 2-3S monomeric form in the presence of strong dissociating agents (4-6M urea) and pH extremes, especially acidic conditions of pH $\leq$ 3.6.

The dissociation-association profile revealed the 12S structure was not stabilized by covalent disulphide bonds. The native form was stabilized by noncovalent interactions, especially entropically driven hydrophobic interactions (Prakash and Rao, 1986). Disulphide stabilization was only observed in the monomeric 2-3S forms. In the presence of a disulphide bond breakers, such as mercaptoethanol, the 2-3S monomer dissociated into acidic and basic subunits (Derbyshire *et al.*, 1976; Schwenke *et al.*, 1983; Mieth *et al.*, 1983a).

2. Quaternary Structure

According to Mieth *et al.* (1983a) and Schwenke *et al.* (1983), the 12S rapeseed protein has a morula structure with an almost spherical shape and a maximum diameter of 11.2nm. The best model consists of six

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


12S
low ionic strength
high ionic strength
7S
high or low pH
or urea
2-3S
reducing agent
basic subunit
acidic subunit

structurally identical subunits, composed of two polypeptide chains, arranged as a trigonal antiprism with dihedral point group symmetry of 32 (Plietz *et al.*, 1983). This model was derived by electron microscopy and small angle X-ray scattering data. The physicochemical properties and structure of the 12S rapeseed globulin are illustrated in Table 2.

3. Secondary Structure

Through the use of circular dichroism measurements, Schwenke *et al.* (1983) determined the secondary structure of the native 12S globulin (Table 1) which does not fit into any of the classical secondary structure classifications. The protein was rich in β-pleated sheets (31%), aperiodic or random coil (58%) and the remaining segments were α-helical (11%) in nature. Since the 12S protein is not important in any biological activities an ordered structure was not necessary (Wright, 1987; Norton, 1989). The role of the large molecular weight protein in the seed was to release a large amount of amino acids during germination. The random orientation of the secondary structure into a spherical shape helps orientate a large number of amino acids into a small area.

4. Subunit Profile

As previously stated the 12S globulin consists of six subunits, each composed of two polypeptide chains. In 1983, Schwenke *et al.* summarized the X-ray scattering data and indicated each subunit consisted of two polypeptide chains joined by a disulfide bond where one polypeptide chain occupied a smaller volume. Derbyshire *et al.* (1976) indicated the basic polypeptide chain was smaller than the acidic chain and showed a smaller basic unit (PPC1 or PPC2) combined with a larger polypeptide chain (PPC3

TABLE 2. Physicochemical properties and structure of 12S rapeseed globulin

Property	Value
Sedimentation coefficient (S°_{20}, 10^{-13} sec)	12.7
Diffusion coefficient (D°_{20}, 10^{-7} cm^2/sec)	3.8
Intrinsic viscosity (η) (mL/g)	
1M NaCl	3.7
0.025 Tris-glycine buffer (pH 8.3)	4.5
Strokes 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
Carbohydrate content (%)	1.2-12
Molecular form	oblate rotations ellipsoid
Dimensions (nm)	
Electron microscopy	11.3 X 11.3 X 9.2
Small angle scattering	10.5 X 10.5 X 9.2
Isoelectric point	7.25
Quaternary structure	trigonal antiprism
Number of subunits	6
Number of polypeptides	12

Adapted from Mieth *et al.* (1983a)
Schwenke *et al.* (1983)
Prakash and Rao (1986)

or PPC4) to form one subunit (2-3S) with a molecular weight of approximately 50,000 (Table 3).

The X-ray scattering data helped explain the gel filtration and electrophoretic observations. In the presence of 8M urea the 12S globulin dissociated to the 2-3S form. Therefore, one band or peak with a molecular weight of 50,000-53,000 was observed in gel filtration or electrophoretic measurements. When the heterogeneous intermediary 2-3S form was exposed to a reducing agent, mercaptoethanol, the acidic component PPC3 with a molecular weight of 26,800 or PPC4 with a molecular weight of 31,200 could be cleaved from either PPC1 or PPC2 with molecular weights of 18,000 or 21,000 respectively.

5. Amino Acid Profile

In the literature, substantial differences in the amino acid content were observed between varieties as well as between seed and meal samples. Commonly, the meal and 12S globulin have a relatively high protein content with a well balanced amino acid composition. A number of researchers have demonstrated the 12S globulin was high in glutamic acid and aspartic acid, with considerable amounts of arginine, but low amounts of sulfur containing amino acids such as cysteine and methionine (Finlayson *et al.*, 1968; Goding *et al.*, 1970; Gill and Tung, 1976; Gill and Tung, 1978a; Schwenke *et al.*, 1981; Prakash and Rao 1986; Norton, 1989). Significant differences in the amounts of sulfur containing amino acids and tryptophan were observed in the literature. This variability could be due to species and cultivar differences or may be due to problems associated with the estimation of the small amounts of these amino acids. Schwenke *et al.* (1981) indicated that argentometric-amperometric titrations yield lower

TABLE 3. Molar mass of the polypeptide chains of the 12s rapeseed globulin after denaturation in the presence of SDS and mercaptoethanol

Polypeptide	Molecular Weight
PPC1	18,000± 800
PPC2	21,000± 500
PPC3	26,800± 900
PPC4	31,200± 1,600

Schwenke *et al.* (1983)

cysteine levels than the Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid). With the Ellman's reagent, Schwenke *et al.* (1981) was able to determine the 12S globulin contained 13 disulphide bridges and 5 thiol groups, both, buried in the interior of the molecule.

When converting from nitrogen to protein Mossé and Pernollet (1983) suggested using a Kjeldahl conversion factor of 5.67. This value is not universally used throughout the literature. As many researchers use a value of 6.25 or 5.85, therefore, consideration should be given to the conversion factor used.

The ratio of acidic to basic residues was found to be 1.0 (Schwenke *et al.*, 1981). This value seems reasonable considering the neutral isoelectric point (7.25) of the 12S globulin.

6. Hydrophobicity

Structural parameters, that depend only on the amino acid composition of the molecule, are Waugh's NPS or frequency of nonpolar side chains, Fisher's P or ratio of the volume occupied by nonpolar residues and Bigelow's average hydrophobicity, based on Tanford's free energy transfer of amino acid side chains from an organic environment to an aqueous environment (Prakash and Rao, 1986). Schwenke *et al.* (1981) used the amino acid composition of the 12S globulin to estimate the average hydrophobicity ($H\phi_{ave}$) to be 1041.2 cal/residue or 4359 J/residues. When comparing the 12S rapeseed globulin with other oilseeds, the rapeseed globulin had the highest the $H\phi_{ave}$ value and nonpolar side chain frequency (NPS) but the lowest polarity ratio (P) (Table 4). Thus, based on any of the structural parameters, the 12S canola globulin was relatively hydrophobic.

TABLE 4. Hydrophobicity values of various 12S plant proteins

Origin	Protein	$H\phi_{ave}$[a]	NPS[b]	P[c]	References
Soybean	Glycinin	944	0.30	1.35	Schwenke *et al.* (1981)
		782	0.30	1.28	Prakash and Rao, (1986)
Peanut	Arachin	860	0.29	1.73	Prakash and Rao, (1986)
Sesame seed	α-Globulin	872	0.26	1.36	Prakash and Rao, (1986)
Sunflower seed	Helianthinin	951	0.31	1.21	Schwenke *et al.* (1981)
		832	0.26	1.25	Prakash and Rao, (1986)
Safflower seed	Carmin	824	0.26	1.69	Prakash and Rao, (1986)
Pumpkin seed		980	0.31	1.32	Schwenke *et al.* (1981)
Rapeseed	Cruciferin	1041	0.36	1.01	Schwenke *et al.* (1981)
		962	0.31	1.03	Prakash and Rao, 1986

[a] Average hydrophobicity according to Bigelow (1967).
[b] Frequency of nonpolar side chains according to Waugh (1954 as cited by Prakash and Rao, 1986).
[c] Polarity ratio according to Fisher (1964 as cited by Prakash and Rao, 1986).

C. Recovery and Purification

1. Extraction

Although canola seed is processed primarily for its oil, the residual meal is a valuable commodity. Oilseed meals have been used as fertilizers in China and Japan, and have long been employed as protein supplements for animal feeds (Girault, 1973; Gill and Tung, 1976; Bruckner and Mieth, 1983; Bell, 1989; Downey and Röbbelen, 1989). Following oil extraction, canola meal contains a high protein content (36-40% protein on dry weight basis) with an excellent amino acid profile (Downey and Röbbelen, 1989). Canola meal has improved palatability and lower glucosinolate levels than rapeseed meal. With steps to further detoxify the meal, it has good potential for being a human food source and canola processors would not have to depend solely upon the use of canola oil for profit.

In the literature, a wide variety of experimental conditions have been utilized during meal processing. Typical extraction solvents are alkali, water, polyphosphates or salt solutions. An early method for preparing protein isolates was to disperse the protein in alkaline solutions and then recover the protein by acid precipitation (Sosulski and Bakal, 1969; Gillberg and Törnell, 1976a; Mieth *et al.*, 1983b). The resulting isolates contained a high protein content but low functionality (Cheftel *et al.*, 1985). Therefore, new methodologies were needed to maintain the high protein content, increase protein yields and decrease the nonproteinaceous material.

With water, polyphosphates and various salt extractions, a variety of solid:liquid ratios, ionic strengths, pH values and temperatures have

been used on a number of genetically different rapeseed and canola defatted meals. The endless combinations of extraction conditions used in the literature makes it difficult to compare different extraction procedures. This discussion will therefore focus on the solubility of protein in water, NaCl solutions and sodium hexametaphosphate (SHMP). Special attention will be paid to the effect of pH, ionic strength and extraction temperature on the protein yield and contaminating factors. Conditions that promote the extraction of the protein and limit the solubility of the antinutritional factors are desired.

The nitrogen solubility of rapeseed meals in various solutions is illustrated in Table 5. In the literature, a wide range of relatively high nitrogen solubilities have been observed in water. Early research by Smith *et al.* (1959), Sosulski and Bakal (1969), and Mieth *et al.* (1983b) demonstrated that 31-52% of the total nitrogen was soluble in water. Schwenke *et al.* (1973 as cited by Prakash and Rao, 1986) extracted proteins from defatted rapeseed flour in water over a pH range of 2 to 6 and the maximum extractability was 50% at any single pH value. Quinn and Jones (1976) reported that the nitrogen solubility of rapeseed meal was 60-65% in water between pH 5 to 8, and then increased up to 90% as the pH increased above pH 8.0. In water, two minimum solubilities were observed: one between 3.7 and 4.0 and one between 7.7 and 8.0. As the pH becomes more alkaline (>8) the high molecular weight proteins become more water soluble. This explains the increased extractable nitrogen above pH 8.0 and why many researchers have used alkaline conditions to extract the 12S protein.

TABLE 5. Nitrogen solubility of rapeseed meals in various solutions

Species	Solvent	solid-liquid ratio	N-soluble (%)	Reference
B. campestris	water	1:50	31.1	Smith *et al.*, 1959
	0.01M NaOH		65.8	
	0.5M Na_2HPO_4		66.0	
	0.6M NaCl		68.8	
B. napus	0.01M $Na_4P_4O_7$	1:30	44.4	Bhatty *et al.*, 1968
	10% NaCl	(3x)	67.0	
B. napus L. Nugget	1M NaCl	1:10		Finlayson *et al.*, 1968
B. campestris polish	water	1:25	44.6	Sosulski and Bakal, 1969
	0.2% NaOH		6.3	
	5% NaCl		24.0	
echo	water	1:25	44.5	Sosulski and Bakal, 1969
	0.2% NaOH		6.6	
	5% NaCl		25.0	
Zero strain	water	1:25	44.7	Sosulski and Bakal, 1969
	0.2% NaOH		5.5	
	5% NaCl		24.7	
B. napus argentine	water	1:25	51.3	Sosulski and Bakal, 1969
	5% NaCl		20.5	
	0.2% NaOH		8.1	
Target	water	1:50	50.6	Sosulski and Bakal, 1969
	0.2% NaOH		9.1	
	5% NaCl		20.5	
Oro	water	1:25	48.4	Sosulski and Bakal, 1969
	0.2% NaOH		8.5	
	5% NaCl		22.4	
B. napus Bronowski	10% NaCl	1:30	70%	Lo and Hill, 1971a
B. napus	10% NaCl	1:20	67	Girault, 1973
	0.1N NaOH		89	
	1.0N NaOH		88.6	
B. napus	1.0M NaCl	1:10	67	Finlayson *et al.*, 1976
	0.1N NaOH		83	
B. napus Bronowski	1.0M NaCl		65	
	0.1N NaOH		84	
B. napus Panter	water	1:20		Gillberg and Törnell, 1976a
	pH 11.1		94	
	8.2		62	
	4.2		57	
B. campestris echo	water	1:20		Quinn and Jones, 1976
	pH 3.7-4.0		58	
	7.7-8.0		80	
	5% NaCl		80	
B. napus oro	2% SHMP	1:10	82	Thompson *et al.*, 1976
	pH 7.0	(2x)		
B. napus	5% NaCl	1:10		Gillberg, 1978
	pH 5.0		50	
	6.0		50	
	8.0		40	
Unknown	2% SHMP	1:10 & 1:6	53%	Liu *et al.*, 1982

Polyphosphates are effective as complexing agents in protein isolation and have been used to extract fish proteins (Spinelli and Koury, 1970) and recover whey proteins (Hidalgo *et al.*, 1973). When using polyphosphates, both the yield and functional properties of the extracted protein improved. Despite these advantages, limited work has been reported on the use of polyphosphates with oilseeds (Table 5). Bhatty *et al.* (1968) extracted only 45% of the rapeseed nitrogen after three extractions with 0.01 M sodium pyrophosphate solution at pH 7.0.

Thompson *et al.* (1976) tried to optimize the processing parameters suitable for the extraction and precipitation of rapeseed proteins using SHMP. At pH 12, 90% of the protein was extracted, however, the extract was undesirably dark. As a result the best extraction solvent was 2% SHMP at pH 7.0 where the nitrogen yield was 82% with a double extraction. After precipitation, by adding an equal volume of water at pH 2.5, the protein yield was 53% (Thompson *et al.*, 1976). The isolate was light in color, as well as low in crude fiber and glucosinolates. However the ash content doubled due to the formation of the hexametaphosphate-protein complex and the phosphorus content increased by 2% (Thompson *et al.*, 1976).

In 1982b, Thompson and associates reported that high ash and phosphorous contents caused enlarged kidneys in rats fed rations containing 20% of the isolate. Furthermore, Lui *et al.* (1982) and Thompson *et al.* (1982b) were concerned that the high levels of phosphates in the effluent would cause economical and pollution concerns.

Using ultrafiltration, diafiltration and ion-exchange with SHMP, Tzeng *et al.* (1988) also extracted rapeseed protein. With 1% SHMP at pH

7 and a solvent to meal ratio of 18, 80% of the nitrogen was extracted. Following ultrafiltration and diafiltration, the protein solution contained 6.29% phytate and 3.61% phosphorous which were further reduced by ion-exchange. The nitrogen yield was 63.1% and the isolate contained 90% protein which was free of glucosinolates, low in phytates (0.86%), phosphorous (0.36%) and fiber, light in color and bland in taste (Tzeng *et al.*, 1988). This study indicated the isolate had good potential for use as a food ingredient.

The literature illustrated that the presence of various electrolytes had a profound effect on the extractability of proteins. A number of researchers have used NaCl to extract protein from rapeseed meal. Using 10% NaCl, Bhatty and associates (1968) and Girault (1973) isolated 67.0% protein while Lo and Hill (1971a) extracted 84%. A wide range of extraction recoveries have been reported with 5% NaCl solutions; Sosulski and Bakal (1969) extracted 20-25% protein while Quinn and Jones (1976) were able to extract 80% of the total protein. After reviewing the literature, Mieth *et al.* (1983b) estimated the protein extractability of 5-10% NaCl solutions to be 66-89%. In other words, a very broad extraction range was observed.

2. Recovery or Precipitation of the Extracted Protein.

After the protein is extracted, it can be recovered using a number of methods to concentrate and purify it. When concentrating the protein a number of procedures may be used including salting out, selective denaturation, solvent removal, isoelectric precipitation, or decrease in ionic strength. To recover the 12S canola globulin, several concentrating approaches have been used alone or in combination. Researchers have

employed ammonium sulfate [$(NH_4)_2SO_4$] fractionation to recover the 12S globulin. Simard *et al.* (1978 as cited by Prakash and Rao, 1986) reported at 25-30% saturation of $(NH_4)_2SO_4$ a 12S protein fraction with more than 90% homogeneity was extracted. Higher percent saturation of $(NH_4)_2SO_4$, preferentially precipitated the 2S protein fraction. The main disadvantage of this method was the high salt levels in the concentrate must be subsequently removed.

When using solvent removal to recover a protein, a number of processes have been employed. After Rao *et al.* (1978) and Lönnerdal *et al.* (1977) used freeze drying, the protein remained along with any electrolytes originally present in the solution. Lacroix *et al.* (1983), Diosady *et al.* (1984), Tzeng *et al.* (1988) and Kroll *et al.* (1989) used ultrafiltration, another solvent removal technique, to recover the 12S canola globulin. Lacroix *et al.* (1983) demonstrated ultrafiltration was also advantageous, because besides removing water, it also removed salts, glucosinolates, nonprotein nitrogen and nitrogen free material. In addition, Tzeng *et al.* (1988) and Kroll *et al.* (1989) reported when using ultrafiltration and diafiltration the amounts of phytate and glucosinolates decreased. Vacuum filtration was used by Tzeng *et al.* (1988) to dry the retentate.

To recover the canola globulins, many researchers have utilized acidic precipitation. However, the solubility curves of the 12S globulin are very complex and greatly depend upon the solvent in which they are dissolved. For instance, when dissolved in 0.1N NaOH, the optimum precipitate is obtained at pH 6.5 (Girault, 1973). In salt solutions (10% NaCl and SHMP), the isoelectric point shifts to a more acidic region

(pH≤3.0) (Girault, 1973). Therefore, an acidic pH promotes protein precipitation. The shift in optimum dissolution or precipitation is influenced by the type of salt and its ionic strength (Gillberg, 1978). In considering salt type, Gillberg (1978) found Cl^- anions strongly increased nitrogen solubility relative to the ClO_4^- anion. The protein's intrinsic amino acids with their wide range of pK_a values also influence protein precipitation. Normally, at pH values ≤3, the protein possesses a net positive charge. However, when a salt is added to the protein solution, the salt ions tend to screen the electric field of the fixed charges on the protein. Thus, the counter-ions would concentrate around the protein and a further decrease in nitrogen solubility would be observed with increasing salt concentrations. A possible explanation was the binding of the negatively charged Cl^- anions to the protein would neutralize some of the positive charges of the protein, weaken the fixed charges in the protein and change the overall charge on the protein thus affecting the protein conformation (Gillberg, 1978).

In the literature, acidic precipitation has been used to recover the 12S globulin (Sosulski and Bakal, 1969; Owen and Chichester, 1971; Girault, 1973; Gillberg and Törnell, 1976a; 1976b; Gillberg, 1978). In addition, this procedure was used by Rao *et al.* (1978) to recover the 2S globulin and albumin after $(NH_4)_2SO_4$ fractionation.

The most prevalent means of recovering the protein is though the reduction of the extracts ionic strength. Bhatty *et al.* (1968) extracted protein in 10% NaCl solutions as well as in 0.01M sodium pyrophosphate mixtures. The extract was then dialysed against water to precipitate the salt soluble nitrogen. Since 1968, this procedure has been implemented by

many researchers to recover salt soluble protein. Lo and Hill (1971a) were able to recover 75-80% of the meal nitrogen after lyophilizing a dialysed salt extract. This procedure was also used by Bhatty *et al.* (1968), Finlayson *et al.* (1968; 1976), Lo and Hill (1971b), MacKenzie and Blakely (1972), and Schwenke *et al.* (1981; 1983).

After solubilizing rapeseed meal in SHMP, Thompson *et al.* (1976; 1982b), looked at the effects of pH and dilution on the precipitation of the extracted nitrogen. The maximum precipitation of the protein in the diluted and undiluted extracts occurred at pH 2.5. Here the shift in the isoelectric point to a more acidic region (pH 2.5) was thought to be associated with changes in acid-base equilibrium within the protein due to the presence of the hexametaphosphate anion (Shimer *et al.* 1973 as cited by Thompson *et al.* 1976). The precipitate increased from 42 to 73% when the ionic strength of the protein extract was reduced with an equal volume of water (Thompson *et al.* 1976).

Murray *et al.* (1981) described a noncovalent approach to extract seed storage proteins where the defatted meal was stirred in a low ionic strength salt solution. Upon dilution of the extract with water, a relatively pure and native protein precipitated to form a viscous, milky white gelatinous mass called the protein micellar mass (PMM). Welsh (1988) used the PMM procedure to isolate canola protein which was light in color, contained minute amounts of antinutritional factors and 78-80% protein. However, the total protein recovery was very low (<5 %). The low ionic strength extraction media (0.1 M NaCl) may have limited the protein solubility. Schwenke *et al.* (1981) explained that canola globulins are readily soluble in high ionic strength NaCl solutions (5%-

10%). Thus increasing the molar concentration of the extraction media could increase the percentage of soluble protein in the extract.

3. Fractionation and Isolation

In fractionating, purifying and isolating the 12S globulin, the chromatographic techniques have been extensively used. Early in the literature, Bhatty *et al.* (1968) resolubilized their precipitate in 5% NaCl at pH 8.5 and applied the sample to a Sephadex G-100 gel filtration column. Approximately 21% of this protein was eluted as the 12 and 17S globulin while 12 % was eluted as the 1.7S protein. Using the above procedure, Bhatty *et al.* (1968), Finlayson *et al.* (1968) and Goding *et al.* (1970) and Gill and Tung (1978a) indicated the fractionation on the Sephadex G-100 resulted in a mixture of both 12 and 17S fractions at the void volume. MacKenzie and Blakely (1972) and Schwenke *et al.* (1981) were able to separate the higher molecular weight fraction from the 12S fraction, using a sephadex G-200 column.

D. Micelle Phenomena

To increase the utilization potential of canola, a variety of protein isolation methods have been developed. One method, the protein micelle mass (PMM) procedure developed by Murray *et al.* (1981), was used by Welsh (1988) to isolate canola proteins. By solubilizing the protein in a high salt / pH controlled environment followed by ionic strength reduction, the insoluble protein micelles can be isolated. This is a mild and selective technique which isolates nondenatured proteins with low levels of antinutritional factors. Since this is such a valuable method, more insight about the protein interactions involved in micelle

precipitation should be gained. The working hypothesis, established by Murray *et al.* (1981) suggested noncovalent forces are of prime importance in plant and food protein utilization with covalent interactions reduced to a secondary stabilizing role. These noncovalent interactions depend upon electrostatic and hydrophobic interactions. As a result, it is important to be able to predict the specific forces promoting the intermicellar protein associations in canola. Understanding the forces involved in the micelle response will help researchers to efficiently and effectively use the PMM procedure to feasibly isolate canola proteins and to formulate structural protein networks which could provide structural integrity in food systems.

1. Molecular Forces in the Micelle Response

Murray *et al.* (1981) demonstrated the usefulness of the micelle response in isolating proteins and producing structural networks. To be able to predict and manipulate the dynamic micelle response, it is important to understand the molecular forces governing the micelle response. In food systems, protein-protein interactions may be the product of covalent and or noncovalent reactions.

Covalent interactions, in protein systems, usually refer to high energy disulphide linkages which form after the protein is in its most thermodynamically stable position (Karp, 1984). Therefore, disulfide bonds generally play a stabilizing role rather than determining the conformation of a protein (Cheftel *et al.*, 1985) and it is not likely these forces play an important role in micelle formation.

In understanding and utilizing proteins in food systems, noncovalent forces are important as they play a determining role in protein structure

(Murray *et al.*, 1981). Noncovalent interactions are fairly well understood in gas or regular solid systems, however, this is not the scenario in the liquid systems in which macromolecules, such as proteins, are studied. Proteins are not frozen into certain conformations. The shape of the energetically preferred structure is a sensitive function of residue composition and sequence as well as the surrounding environment (von Hippel and Schleich, 1969). By subjecting proteins to different environmental conditions the role of noncovalent forces in conformational motility can be observed. Categorically, noncovalent forces are van der Waal forces, hydrogen bonds, electrostatic or ionic associations and hydrophobic interactions.

Cheftel *et al.* (1985) indicated van der Waal interactions are weak electrostatic interactions (typically 1-9 kJ/mole) existing between close atoms in proteins. The attractive or repulsive nature of the interaction is determined by the distance between the atoms and the torsion angles (ϕ and ψ) around the α-carbon. An attractive force develops as the distance between atoms decreases, while the repulsive force develops at a close critical distance. The small net stabilizing effect of van der Waal interactions prevails only at short distances. As a result, these forces play no special contribution to protein structure (Damodaran and Kinsella, 1982).

A hydrogen bond links an electronegative atom (nitrogen, oxygen or sulphur) to a hydrogen atom covalently bonded to another electronegative atom (Creighton *et al.*, 1988). These weak electrostatic interactions may appear between the oxygen of the carboxyl group of the peptide bond and the hydrogen of the amino group of another peptide bond. Hydrogen bonds

stabilize secondary structures such as α-helix and β-sheets, and tertiary structures (Cheftel *et al.*, 1985). These bonds require close interaction distances and provide low to moderate bond energies (8-40 kJ/mol). Therefore, the net contribution of the bond to molecular stability has been questioned. Kollman and Allen (1972) revealed that hydrogen bonded structures are thermodynamically unstable in aqueous conditions. Thus, these bonds may not dramatically influence micelle formation and association.

Proteins are considered polyelectrolytes since the ionizable groups from the amino acid side chains, carboxyl and amino terminals participate in the acid-base equilibrium (Cheftel *et al.*, 1985). Complex titration curves result because each amino acid has its own pK value that is influenced by the surrounding environment, proximity of other ionized groups, hydrophobic residues or hydrogen bonds.

Stronger electrostatic interactions, attractive or repulsive forces, with energies in the magnitude of 42-82 kJ/mole can also occur in protein-water interactions. Attractive forces (salt linkages or ion-pair bonds) occur between closely spaced (2-3Å) residues with a fixed charge of the opposite sign while repulsive forces prevent similarly charged residues from contacting each other (Cheftel *et al.*, 1985). These stronger electrostatic interactions contribute to the stabilization of secondary or tertiary structures, but their role in stabilizing protein structure or micelle response is not easily understood. Even though these electrostatic forces are important in maintaining protein structural integrity, within polar aqueous systems these forces lack the energy required to stabilize proteins (Nakai and Li Chan, 1988).

Hydrophobic interactions are thought to be the entropic driving force for micelle formation. Although individual energies (4-12 kJ/mole) of the interaction are low, the overall reaction energy of the system is generally large because of the involvement of numerous nonpolar groups. Strong interactions between water molecules, rather than a direct interaction between solute molecules are the result of the stabilizing hydrophobic effect. An unfavourable decrease in entropy arises when nonpolar residues are introduced into water (Kauzman, 1959). By increasing hydrogen bonding between water molecules, the water around the solute molecules becomes more ordered and forms a cavity. This scenario requires high energy levels and is thermodynamically unfavourable. To lower the free energy of the system, a majority of the nonpolar residues are internally buried to reduce the surface area of the protein. Therefore, hydrophobic interactions are important in determining protein structure but are also responsible for the formation of protein micelles.

2. Micelle Formation

Tanford (1973) defined micelles as aggregated products that form when amphiphilic molecules are dissolved in aqueous environments. This amphiphilic nature of proteins with numerous exposed nonpolar groups encourages micelle formation in aqueous environments because nonpolar to solvent interactions are not thermodynamically favored. A number of factors are important in elucidating micelle production. Firstly, a critical micelle concentration (CMC) must be reached (Reynolds, 1979) where protein monomers assemble into aggregates and form micelles. Further increases in protein concentration cause more micelle assemblies to form. Secondly, Tanford (1973) indicated that not all proteins can

form micelles as the amount and type (aliphatic or aromatic) of hydrophobic residues are critical factors. Knowing the amino acid composition and hydrophobicity of the protein could help identify proteins with good micelle forming capacity (Bigelow, 1967). Proteins with high numbers of hydrophobic amino acid residues generally have potential for micelle formation (Ismond, 1984). Fisher (1965) found hydrophobic patches on the protein surface. Hayakawa and Nakai (1985) suggested aromatic residues were not as easily buried inside the protein due to their bulky structure. In 1988, Nakai and Li Chan concluded some hydrophobic residues were able to be positioned on the surface while others remained buried inside the protein. Thus, Ismond (1984) proposed if a protein molecule did not possess sufficient polar residues on the surface to bury the hydrophobic patches, they would remain on the surface. 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.

A critical amount of aqueous solvent will be required to bury the hydrophobic groups, however, too much water will dilute the protein and prevent protein interactions. Thus, the dilution factor will also influence the type of micelle response observed.

3. Intermicellar Associations

In addition to the above critical parameters required for micelle formation, noncovalent forces also play a dominant role in determining association between micelles. The research by Ismond *et al.* (1986a; 1986b) and Georgiou (1987) reinforced the original premise that

hydrophobic interactions facilitated the precipitation of micelles (Murray *et al.*, 1981). However, when considering micelle interactions, molecular surface properties of the micelle play a significant role. A specific distribution of the hydrophobic and hydrophilic residues must exist to allow hydrophobic attractive forces to predominate over electrostatic repulsive forces. Two micelles in close proximity, with hydrophobic patches, tend to unite when correctly orientated if the attractive forces predominate over the repulsive electrostatic forces. An increase in electrostatic repulsion could lead to separation of micelles, localized protein unfolding or prevention of coalescence. With increased distances between exposed hydrophobic groups, intermicellar hydrophobic interactions would be limited. This indicates a delicate balance of hydrophilic-hydrophobic noncovalent forces is critical for micelle formation and subsequent association.

The surrounding environment plays a dramatic role in micelle formation and association. The concentration and type of ions as well as the pH of the media influence the conformational properties of the protein and ultimately affect micelle formation and subsequent interaction. The environment determines the charge and orientation of the amino acids and the surface properties of the protein. As a result, the environment and the nature of the protein play critical roles in determining the CMC, size and distribution of micelles, size and number of aggregates, and the texture, regularity and size of the interior micelles of protein networks or sheets. It is essential researchers familiarize themselves with their specific protein and how its environment will influence micelle formation and association. This knowledge will be a factor in successfully

predicting and manipulating the micelle response in food systems.

4. Isolation of Protein Using Micelles

Seed globular proteins have been isolated using a protein micellar mass (PMM) procedure developed by Murray *et al.*, (1981). The PMM procedure involved stirring defatted meal in a low ionic strength salt solution and then diluting the high salt protein extract (HSPE), containing the solubilized protein, with cold water. This sudden decrease in ionic strength combined with low water solubility of the globular protein resulted in protein aggregation, micelle formation and interaction, and precipitation of the protein. The isolated protein was light in color, and relatively free of antinutritional factors (Table 6). Using this procedure, Arntfield *et al.*, (1985) were able to recover 42.5% of the protein from fababean; however, Welsh (1988) recovered less than 5% protein from canola. Since canola proteins have a relatively high water solubility, some of the protein may remain solubilized in the water used to dilute the HSPE. Although this procedure still has potential for isolating canola globulins, researchers must manipulate or adapt the experimental conditions to correspond with the physico-chemical properties of the canola proteins.

5. Effects of Environmental Manipulation on Micelle Formation and Association

To date, fababean has been the study system for increasing awareness of the forces involved in the micelle response. This research forms a structural framework with which to build upon, but cannot be directly applied to canola proteins as they are uniquely different from fababean proteins. Canola proteins possess an isoelectric point close to

TABLE 6. Level of antinutritional factors at various stages in a fababean protein isolation procedure

Stage	TUI[2]/mg	HU[3]/mg	Phenolics (%)	Phytic acid (%)
Concentrate	26.11[a]	8.9	1.44	3.99[a]
High salt protein extract	25.98[a]	10.9	1.15	3.78[a]
Protein isolate (PMM)	5.91	1.0	0.18	0.32[b]

[1] Column numbers followed by the same letter are not significantly different (P 0.05).
[2] TUI-trypsin units inhibited
[3] HU-hemaggultinin

Arntfield *et al.* 1985

neutrality rather than in the acidic range of most plant proteins. Thus, the pH range (6-8) investigated by Ismond *et al.* (1986b) would span the isoelectric point and the net charge on the protein would differ significantly. In addition, canola globulins are capable of dissociating, thereby changing the protein conformation could have a dramatic effect on the micelle response. Even with these significant differences, the research by Ismond *et al.* (1986a; 1986b) and Georgiou (1987) is a beginning for increased awareness of the micelle response of plant proteins and the research could be extrapolated to canola proteins.

a. Effects of pH on Micelle Formation. The effect of pH on the protein micelle response of vicilin, a storage protein from fababean, was investigated by Ismond *et al.* (1986a). A strong micelle response was identified from pH 6.0 to 6.8. As the pH increased above this range, the net negative surface charge stressed the structure, produced conformational changes in the native protein and prevented further micelle association. In addition, a decrease in surface hydrophobicity (So), indicated a reduction of exposed nonpolar residues at higher pH values resulting in fewer micelle interactions.

b. Effects of Neutral Salts on Micelle Formation. To further elucidate the relative importance of noncovalent forces, especially hydrophobic interactions, in micelle interaction, it was necessary to use salt environments to manipulate surface hydrophobicities. Neutral salts were defined by von Hippel and Schleich (1969) as strong electrolytes which are soluble in water without bringing about a major change in solution pH. These salts can affect electrostatic interaction and have a striking

effect on protein conformation. The effect of salt on protein conformation and micelle response was determined as a function of concentration and type of salt.

To examine the influence of hydrophobic interactions on micelle formation, various stabilizing and destabilizing sodium associated anions were selected by Ismond (1986b). The destabilizing or chaotropic anions (NaSCN and NaI) were characterized by a low capacity to increase surface tension of water, high So values, low thermal denaturation temperatures (Td), low enthalpies of denaturation (ΔH) and low micelle responses. Bull and Breese (1970) stated that destabilizing anions bind directly to the protein surface and cause conformational disturbances. This preferential binding would increase the electrostatic and repulsive forces of the protein (Arakawa and Timasheff, 1982). As destabilized proteins unfolded and their solubility increased, lower Td values were observed and the hydrophobic-hydrophilic balance required for micelle formation was disrupted because more nonpolar residues were exposed and the distance between the hydrophobic residues increased. Since chaotropic salts increase the exposure of hydrophobic residues, as indicated by very high So values, they do not provide an ideal environment for micelle formation or interaction.

Highly stabilizing or nonchaotropic salt environments ($Na_3C_6H_5O_7$ and Na_2SO_4) were also unsuitable for micelle responses. Nonchaotropic salts promote preferential hydration (Arakawa and Timasheff, 1982) where water accumulates and salt remains away from the protein. This increases hydrogen bonding and surface tension of the protein environment, and decreases the entropy of the system. Since all systems strive for low

free energy values (von Hippel and Schleich, 1969), the proteins aggregate to decrease the overall area of the cavity and the free energy of the system. This tightly folded protein structure, as indicated by the high thermal denaturation values, enhanced intramolecular hydrophobic interactions. In the presence of nonchaotropic salts, Ismond and associates (1986b) reported low So values were inadequate to promote extensive hydrophobic associations between micelles because the nonpolar residues were buried within the protein molecule. As a result, small discrete micelles formed. Therefore, it appeared the availability of hydrophobic residues was critical to initiate self association between micelles where electrostatic repulsion was minimal.

The research conducted by Ismond *et al.* (1986b) and Georgiou (1987) indicated moderately stabilizing salts ($NaC_3H_6O_7$, NaBr, and NaCl) produced highly intrinsic networks where the hydrophobic-hydrophilic forces were balanced.

Since NaCl is a moderately stabilizing salt, and produces a strong micelle response, it was used as the desired environment of this present research. The effect of NaCl concentration on conformational parameters and the micelle response will be examined more specifically. As the NaCl concentration increased, so did the conformational parameters (Td and ΔH), however, the So value decreased. When considering the effect of NaCl concentration, the micelle response was strong at 0.2M and 0.5M, but significantly decreased in 1.0M and 2.0M solutions.

At low electrolyte concentrations (μ <0.5), the anions act as a collective group and stabilize the protein by nonspecific electrostatic interactions (von Hippel and Schleich, 1969 and Ismond *et al.*, 1986b).

The ions react with the charges on the protein and decrease the repulsive electrostatic attraction between the opposite charges of the neighbouring molecules. As a result, the solubility of the protein was increased. As previously indicated, electrostatic influences predominate at low ionic strengths (μ<0.5). Upon dilution, the sudden reduction in ionic strength would disturb the electrical double layer around the protein, thereby minimizing the electrostatic influences on the micelle surface. Thus, the reduction in repulsive forces would increase potential of the environment for micelle association. At high salt concentrations ($\mu \geq 0.5$), the electrostatic effects become negligible and the protein structure is determined by the lyotropic effect (Ismond *et al.*, 1986b). Consequently, at 0.5M salt levels, the chloride ion would exert a moderately stabilizing influence. Ismond *et al.* (1986b) revealed chloride ions have a tendency of binding to proteins and undermining preferential hydration. Therefore, both, water and chloride ions would accumulate around the protein. This electrostatic salt binding could affect hydrogen bonding between the water and protein, weaken fixed protein charges, and affect protein conformation (Gillberg, 1978 and Ismond *et al.*, 1986b). The electrostatic interactions between the charged residues could be neutralized by the chloride ion shield and electrostatic interactions would become less important than at <0.5M. This neutralizing electric field also limits interaction between the water and protein or preferential hydration and results in fewer hydrophobic residues forced away from the aqueous shield. Thus, the So values do not decrease as rapidly as observed with highly stabilizing anions ($Na_3C_6H_5O_7$ and Na_2SO_4) at 0.5M concentrations. As in the 0.2M environment, the physical impact of the aqueous solvent has disrupted the

electrical double layer associated with the protein, allowing the thermodynamically favorable aggregation of hydrophobic residues and micelle association.

At high NaCl concentrations (≥ 1.0M), the So value of 210 seemed adequate for micelle formation, however the strong micelle network disintegrated immediately. Under these conditions, the binding of the chloride ion increased electrostatic repulsions resulting in a deterioration of the protein association.

The research by Murray *et al.* (1981), Ismond *et al.* (1986a; 1986b; 1989) and Georgiou (1987) indicated environmental solvents can manipulate surface properties of the dynamic, and flexible proteins to form micelles. Moderately stabilizing salts, specifically NaCl, produce desirably strong micelle responses. In addition, since NaCl is inexpensive, readily available and a recognized food ingredient, it was selected as the prime milieu for this study. The micelle response will be manipulated by changing the pH and ionic strength of this environment with hopes of gaining insight on how to adapt the PMM procedure for canola proteins. In performing this research, conditions such as protein concentration, dilution ratio, pH and ionic strength required for optimum micelle precipitation may be recognized and applied to the PMM procedure. This research is also important, to better understand the environments required for the production of three dimensional networks. This will provide some guidelines on how to manipulate the micelle response to form a structural framework needed for many different food systems.

III. MATERIALS AND METHODS

A. Preparation of Canola Meal

Defatted commercial canola meal was obtained from CSP Foods in Altona, Manitoba. Approximately 500g of the commercial meal was ground in a Micro-mill (Chemical Rubber Company in Cleveland, Ohio) to a maximum particle size of approximately 425μm.

1. Proximate Analysis

a. Moisture Content Determination. The moisture content was determined using a vacuum oven as described in the AOAC official Methods (AOAC, 1975). Aluminum dishes were pre-dried in a air oven for 2h at 100°C and then cooled in a desiccator for 10 minutes. The ground defatted canola meal was mixed and 2g samples were accurately weighed into the pre-dried dishes. The samples were dried in a vacuum oven at 100°C for 18h and after cooling in a desiccator the samples were accurately weighed on an analytical balance. The moisture content was determined by the weight loss times 100 divided by the original sample weight.

b. Protein Nitrogen Determination. The protein content of the ground, dried, defatted canola meal was determined by microkjeldahl analysis as outlined in the AOAC Official Methods of Analysis (AOAC, 1975) Section 47.021. The calculations used for estimating the protein content was as follows:

$$\frac{[(\text{mL HCl}) - (\text{mL blank}) \times \text{normality of HCl} \times 14.007 \times 100]}{\text{mg sample}} \times 5.67$$

The protein to nitrogen conversion factor for canola used was 5.67 as suggested by Mossé and Pernollet (1983).

c. Crude Fat Determination. Crude fat was estimated according to AOAC Official Methods of Analysis (AOAC, 1975) using hexane to extract the fat. Flat bottom 250mL flasks containing 3-5 glass beads were pre-dried for 1h at 125°C in an air oven. The flasks were allowed to cool for 30 minutes in a desiccator and were then weighed on an analytical balance. Using thimbles, three samples of ground, dried and defatted canola meal ranging from 3-4g were accurately weighed, recorded and coded. Glass wool was used to cover the sample in the thimble and 150mL of hexane was added. With the Soxhlet apparatus set up, the sample was refluxed for 16h. After refluxing, a steam bath was used to evaporate the flasks to dryness. The flasks were then placed in an air drying oven for 1h at 100°C, allowed to cool in a desiccator for 1h and the weighed on an analytical balance. The percentage of fat loss was determined by the weight loss after extraction divided by the original sample weight and this value was multiplied by 100.

d. Total Ash Determination. Ash crucibles were pre-ashed in a muffle furnace at 550°C for 1h, allowed to cool in a desiccator for 1h and then weighed on an analytical balance. Three samples of ground, dried, defatted canola meal (3 ± 1g) were accurately weighed in each crucible. Each sample was pre-ashed in an open crucible for at least 20 minutes. The crucibles were placed into a muffle furnace at 550°C for 18h, then

cooled in a desiccator and weighed. Drops of distilled water were applied to the ash which was again placed in the furnace for 24h at 550°C, cooled and then weighed. The above procedure was performed in triplicate. The total ash content was determined by the weight loss of the sample divided by the original sample weight and this value was multiplied by 100.

e. Phenolic Determination. The phenolic components were extracted using a modification of the procedure used by Dorrell (1976). Oven dried, defatted and ground canola meal samples weighing 50mg were refluxed for 30 minutes in 12.5mL of 80% ethanol at pH 4.0. The samples were cooled and then centrifuged in a Sorvall GLC-1 benchtop centrifuge for 5 minutes at 559xg. The decanted supernatant volume was adjusted with distilled water to 25mL.

The methodology of Swain and Hillis (1959) was utilized to assay the phenolic components in the supernatant. However, as suggested by Schanderl (1970), the Folin-Ciocalteu reagent was used instead of the Folin-Denis reagent used by Swain and Hillis (1959). In a 15mL graduated test tube, a 500μL aliquot of the supernatant was diluted to 7mL with distilled water and vortexed. To the test tube, 500μL of the Folin-Ciocalteu reagent was added, vortexed and allowed to stand for 3 minutes. Then one millilitre of saturated sodium carbonate solution was added to the tube, then distilled water was added until the total volume reached 10mL and the solution allowed to stand at room temperature for 1h. Triplicate samples and tannic acid standards of 50, 100, 150, and 200μg/mL were treated in a similar manner and read at 750nm in a LKB Ultrapec II, model 4050. Distilled water was used as a blank. The phenolic content

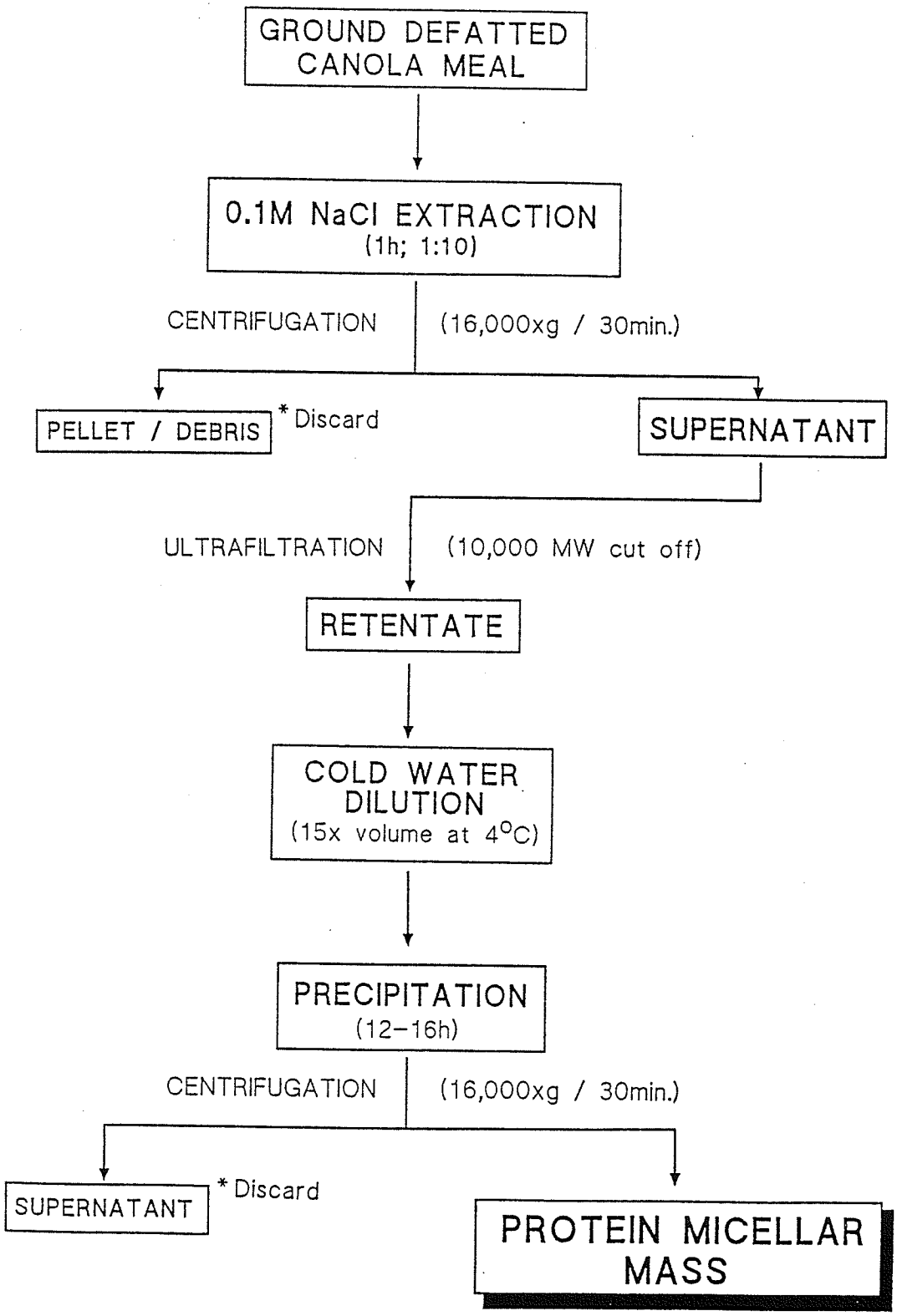
was determined from the linear regression of the tannic acid standards (r^2=0.998, y=0.003x - 0.011). These values were converted to mg/mL, divided by the original meal sample size and multiplied by 100.

f. Phytic Acid Determination. The phytic acid content was determined on ground, defatted canola meal using the methods described by Latta and Eskin (1980). The assay was performed in triplicate.

B. Isolation of Canola Protein by Protein Micellar Mass Procedure

Initially, the canola protein was isolated based on the methodology of Welsh (1988) as illustrated in Figure 2. This procedure was slightly modified with the hopes of increasing the protein yield. Ground meal samples weighing 500g were stirred overnight (8-10h) in 5000mL of 0.1M NaCl, then centrifuged in 1L centrifuge bottles for 30 minutes at 150,000xg using a Sorvall refrigerated centrifuge, model RC-3. The cellular debris or precipitate settled firmly to the bottom of the centrifuge bottle allowing the supernatant to be more easily decanted from the cellular debris than was possible in the 250mL centrifuge bottles. The high salt protein extract or supernatant containing the solubilized protein was filtered through four layers of pre-moistened cheese cloth and then concentrated through a 10^4 molecular weight cut off HIP10-20 Diaflo Hollow Fiber Cartridge in an Amicon Hollow Fiber Dialyser/Concentrator, model DC-2. The pressure was maintained at 30 units on the pressure gauge with the volume of the supernatant being reduced at least eight times. The solubilized protein concentration was diluted by six times its volume with refrigerated distilled water. The

Figure 2. A flow diagram of the modified micellar mass (PMM) process used by Welsh (1988) to isolate canola proteins



(Adapted from Welsh, 1988)

solution was then refrigerated at 4-8°C, to allow the micelles to precipitate to the bottom of the dilution vessel overnight.

The following day, approximately 10-16h later, the protein micelles were recovered by centrifugation using a Sorvall Refrigerated centrifuge, model RC2-B with a GSA rotor head, at 150,000xg. After 30 minutes centrifugation, the decanted supernatant was discarded and the protein precipitate was collected and frozen at -40°C.

C. Solubility Assessment of Commercial Canola Meal

1. Soluble Protein Determination

The concentration of the soluble protein was evaluated by the Lowry *et al.* (1951) and the Coomassie micromethod (Pierce Chem. Co., 1986). Bovine Serum Albumin was obtained from Sigma Chemical Co. and used as a standard.

2. Effect of NaCl Concentration, Meal to Solvent Ratio, Time on Protein Extractability of Commercial Canola Meal

Since canola proteins were highly soluble in NaCl and NaCl is a common food constituent, it was used to further optimize the extraction of the major canola protein. A number of parameters can effect the solubility of the canola meal in NaCl solutions including the ionic strength or NaCl molar concentration, exposure time and ratio of meal to NaCl solution. To monitor the influence of NaCl, the molar concentration of NaCl increased by 0.05 units from 0.05 to 2.0 and then by 0.025 unit up to 2.0M.

Five grams of ground meal was dissolved in 50 (1:10) or 100 (1:20) mL of solvent and then stirred for 1,2,or 4h. After the appropriate time,

the pH and conductivity (measured by a CDM83 conductivity meter) of each solution was monitored to ensure reproducibility. The mixture was then centrifuged for 30 minutes at 12,000xg using a model RC-3 Sorvall refrigerated centrifuge and the supernatant was decanted through four layers of cheesecloth. The pH, volume and solubility of the supernatant and the percent recovery of the protein were determined. Each extraction was performed at least in duplicate.

3. Effect of Sodium Hexametaphosphate Concentration on Protein Extractability of Commercial Canola Meal

In the research preformed by Thompson *et al.* (1976, 1982) and Tzeng (1988), sodium hexametaphosphate (SHMP) was found to complex with canola proteins and have a profound effect on their extractability. Thus, the solubility of the canola protein from this meal in various concentrations of SHMP was also investigated. In this instance, the protocol for solubilizing the protein was designed to duplicate the basic extraction procedure.

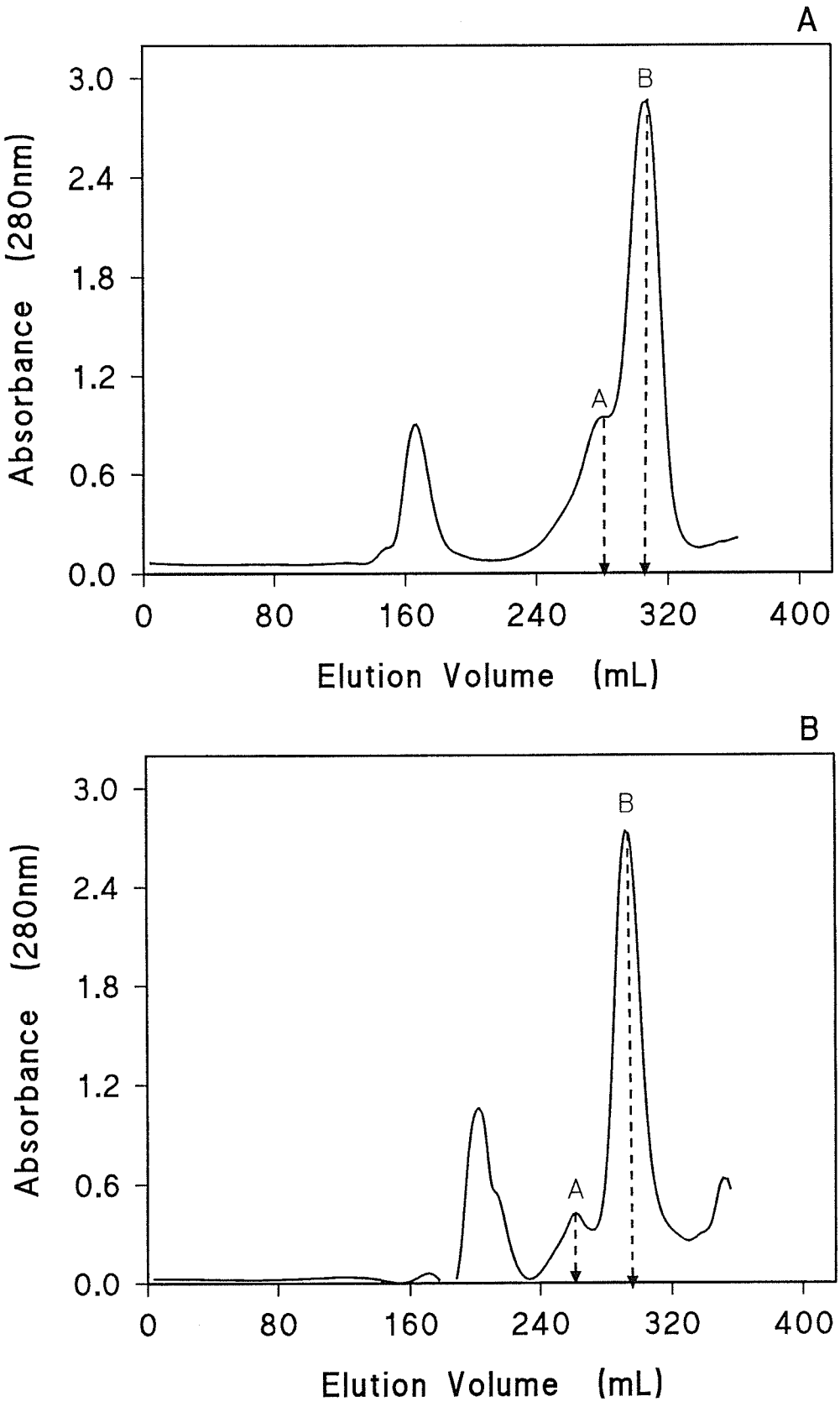
A 5g meal sample was mixed with 50mL of SHMP at the following concentrations (w/v): 0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0%. After the dispersion was stirred at room temperature for two hours in a fumehood, the pH of the dispersion was measured. Following 30 minutes of centrifugation in a Sorvall refrigerated centrifuge at 12,000xg, the supernatant was collected after passing though four layers of premoistened cheesecloth. The pH was remeasured and the volume and protein solubility of the supernatant were determined. Each extraction was performed at least in duplicate.

D. Extraction and Purification of the Major Storage Canola Protein

Based on the solubility data, the 0.5M and 2.0M NaCl solutions extracted similar levels of protein. As seen in Figure 3a and b, the protein elution profiles were the same for both the 0.5M and 2.0M NaCl Sephacryl S-300 columns. However, when running 2.0M NaCl through the column, the sephacryl matrix compacted and extremely long retention times were noted. Due to these problems, further extractions with 2.0M NaCl were eliminated. Therefore, when extracting and purifying the major canola protein, samples weighing 1.5g were stirred for 2h in 10mL of 0.5M NaCl. The mixture was then centrifuged for 30 minutes at 12,000xg in a Sorvall refrigerated centrifuge, model RC-3. To eliminate the cellular debris, the supernatant was filtered through 5.5 cm Whatman #1 filter paper, pre-moistened with 0.5M NaCl and 0.02% (w/v) sodium azide. The protein concentration of this filtered supernatant was determined.

To collect the major storage protein, approximately 7mL of the supernatant was applied to a calibrated Pharmacia K26-100 gel filtration column containing Sephacryl S-300. The ionic strength of the 0.5M NaCl and 0.02% (w/v) sodium azide solution ranged from 42-48mS/cm, while the pH was approximately 5.9. The flow rate of the salt solution onto the column was 30mL/h which was maintained by a LKB Microperpex peristaltic pump, model 2132. A LKB Redirac fraction collector, model 2117, collected 85 drops per tube or approximately 3.76mL while the absorbance of each test tube was monitored on a LKB Ultraspec II, model 4050. A typical absorption curve is illustrated in Figure 3a. The prominent fractions of the main peak (B) in Figure 3a were pooled and concentrated in an Amicon Ultrafiltration unit containing a PM10 membrane with a 10,000 molecular

Figure 3. Typical elution profiles of the major salt soluble proteins when applied to the Sephacryl S-300 gel filtration columns.
A. The 0.5M NaCl extract applied to the column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v)
B. The 2.0M NaCl extract applied to the column equilibrated with 2.0M NaCl with 0.2% sodium azide (w/v)


A
B
3.0
2.4
1.8
1.2
0.6
0.0
Absorbance (280nm)
A
B
0
80
160
240
320
400
Elution Volume (mL)
B
3.0
2.4
1.8
1.2
0.6
0.0
Absorbance (280nm)
A
B
0
80
160
240
320
400
Elution Volume (mL)

weight cut-off and the pressure of the system was maintained at 55 psi. The volume of the supernatant was concentrated from 120 to 12mL to obtain a protein concentration of approximately 5-8mg/mL.

E. Characterization of the Soluble Canola Protein

1. Molecular Weight Determination

Schwenke *et al.* (1981) described an association-dissociation profile for the 12S canola globulin. To determine if the various molecular forms had any impact on micelle formation, it was important to monitor the molecular weight of the protein using several different techniques in various environments.

a. Gel Filtration. The Pharmacia K26-100 columns with Sephacryl S-300 matrix equilibrated with either 0.5M or 2.0M NaCl were calibrated with approximately 36mg of lyophilized Bio-Rad Gel Filtration Standard dissolved in the appropriate salt solution. The standard mixture contained the molecular weight markers indicated in Table 7. The flow rate of the salt solution onto the column was 30mL/h which was maintained by a LKB Microperpex peristaltic pump, model 2132. An LKB Redirac Fraction collector detected 85 drops of eluent per tube. Using an LKB Ultraspec II, model 4050, the absorbance of each tube was measured at a wavelength of 280mn. The molecular weight of the soluble canola proteins were determined from the linear relationship between the $\log_{10}$ molecular weight and elution volume of the standard proteins (Appendix A and B).

In the various salt environments, purified and environmentally manipulated samples were applied to a HiLoad 16/60 Superdex 200 prep grade Pharmacia gel filtration column to determine the molecular weight of the

TABLE 7. Molecular weight of bio-rad protein standards

Protein	Molecular weight	Log_{10} molecular weight
Vitamin B-12	1,350	3.130
Myoglobin (horse)	17,000	4.230
Ovalbumin (chicken)	44,000	4.644
Gammaglobulin (bovine)	158,000	5.199
Thyroglobulin (bovine)	670,000	5.826

canola proteins. This column is chemically stable over a wide pH range and with strong salt solutions and the column was equilibrated with 0.5M NaCl pH 6.0 and later with 0.5M NaCl pH 4.0. The operating conditions were similar to the procedures used with the Pharmacia K26-100 columns.

When calibrating the Superdex column, the Pharmacia standard proteins from the calibration kit were run in three separate groups to ensure resolution of their peaks for accurate elution volume measurements. Blue Dextran 2000 with a concentration of 1mg/mL was applied in the first run. In the second run, thyroglobulin (2mg/mL), catalase (5mg/mL) and bovine serum albumin (7mg/mL) were eluted through the column. In the final run, ferritin (0.5mg/mL), γ-globulin (2mg/mL; Sigma) and ovalbumin (7mg/mL) were applied. After reading the absorbance of each tube on a LKB model 4050 Ultraspec II and determining the elution volumes, a calibration curve was prepared (Appendix C and D). The experimental protein samples were then eluted through the column and the molecular weights of the soluble canola proteins were determined from the calibration curves.

b. Gradient Polyacrylamide Electrophoresis. Molecular weight determination by liquid chromatography are carried out by comparing elution volumes of calibration proteins with known $\log_{10}$ molecular weights. However, liquid chromatography techniques do not directly determine molecular weight values. As a result, several unsuccessful attempts were made to find running ultracentrifugation equipment with Schlieren optics or molecular weight determining capabilities. After personal communications with Dr. LeJohn an accurate but still indirect method, gradient polyacrylamide electrophoresis, was used to also assess the

molecular weights of the salt soluble canola proteins.

Gradient polyacrylamide electrophoresis was preformed as described by Dr. LeJohn in personal communications. This procedure was performed on a Bio-Rad Protean dual slab vertical electrophoresis cell along with a Bio-Rad model 3000/300 power supply and a Haake F3 digital circulating water bath set at 20°C. A gradient gel with an acrylamide range of approximately 3% to 19.6% was prepared by mixing the components in the proportions given in Table 8 in a gradient mixer. The gel was allowed to polymerize for 1h at room temperature with 95% ethanol on top of the gel. After polymerizing a 4% stacking gel was applied on top of the gel by blending the components in Table 9. For easier sample application the density of the protein solutions were increased with 40% sucrose (w/v) as suggested by Pharmacia (1982b). These samples, with protein concentrations of 2mg/mL and 1mg/mL, and the Bio-Rad Molecular weight standards were applied to the gel. The gel was pre-electrophoresed at 10mA/gel for 2h and electrophoresis was carried out at 20mA/gel for 26h. The gels were stained for 1h and destained for 24h in the buffer solutions indicated in Table 10. The gels were then wrapped in Biowrap (Bio Design Inc., New York), the migration distance of the standards and protein bands were measured. From the linear relationship of migration distance as a function of $\log_{10}$ molecular weight of the standards, the molecular weights of the protein bands were determined (Appendix E). The gels were then photographed using Kodak Ektachrome slide film (160 tungsten).

Three gels were run following the procedure described above where the protein samples were obtained from the 0.5M NaCl Sephadex S-300 gel filtration column. As previously mentioned, the pooled fractions were

TABLE 8. Composition and volumes of solutions required to make a gradient (3.0% to 19.6%) polyacrylamide gel

	Solution		Solution volume (mL) Gel conc.	
			3%	19.6%
A.	48mL 36.3g 0.23mL	1N HCL Tris TEMED Make up to 100mL with distilled water. Adjust the pH to 8.9 with 1N HCL.	2	2
C.	28g 0.735g	Acrylamide Bisacrylamide Make up to 100mL with distilled water. Filter with Whatman No. 1 filter paper.	2	18
D.	water		15.9	0
E.	10%	Ammonium Persulfate	0.16	0.16

TABLE 9. Composition of the 4% stacking gel used in gradient page

	Solution		Solution Volume (mL)
B.	48mL 5.98g 0.46mL	1 HCL Tris TEMED Make up to 100mL with distilled water. Adjust pH to 6.7 with 1N HCL.	1.25
C.	28g 0.735g	Acrylamide Bisacrylamide Make up to 100mL with distilled water. Filter with Whatman No. 1 filter Paper.	0.70
D.	Water		3.00
E.	10%	Ammonium Persulfate	0.08

Table 10. Composition and application time of the solutions used in gradient page

Solution	Time	Solution Composition	
Stock Barbital Buffer (pH 8.7)	-[a]	10g	barbital acid
		30g	Tris
		4mL	0.5M EDTA pH 8.0
			Check pH and adjust volume to 200mL with distilled water.
Staining	2h	2.5g	Coomassie Brilliant Blue R-250 (Sigma Chem. Co.),
		450mL	distilled water.
Destaining	24h	450mL	methanol
		90mL	acetic acid

[a] Running Time for Gradient electrophoresis (24-36h).

concentrated and exhaustively dialysed with 0.05, 0.1, and 0.3M NaCl solutions with 0.02% sodium azide. The three samples along with a 0.5M NaCl protein sample were concentrated to 1mg/mL and 2mg/mL and applied to the gels. A third run was performed, where proteins fractions (shoulder A and main peak B) were collected from the 0.5M NaCl Sephadex S-300 gel filtration column (Figure 3a) and were examined using gradient PAGE. In addition, the voltage for the pre-electrophoresis was increased to 20mA/gel and electrophoresis voltage was increased to 30mA/gel.

2. Isoelectric Point Determination by Isoelectric Focusing

The methodology Winter and Anderson (1977) described in the LKB Application note number 250 was utilized to determine the isoelectric point of the major canola protein. Due to the inhibitory effect of salts on good band resolution, the protein samples in 0.5M NaCl were dialysed in 0.3M, 0.1M and 0.05M NaCl solutions. To prevent protein precipitation of the protein samples, the ionic strength was not reduced below 0.05M NaCl. For the four NaCl solutions (0.5, 0.3, 0.1 and 0.05M NaCl), the protein content was diluted to both 1mg/mL and 5mg/mL with the respective buffer giving eight samples for isoelectric point determination.

Isoelectric focusing of the eight samples was carried out on an LKB 2117 Multiphor apparatus with an LKB 2197 Constant Power Supply. To control the temperature at 10°C a Haake circulating water bath was used. A LKB Ampholine PAG plate polyacrylamide gel with a pH range of 3.5 to 9.5 was used. After the PAG plate was in place, one electrode strip soaked in 1M H_3PO_4 was positioned at the anode while a 1M NaOH solution was used to soak the electrode strip situated at the cathode. Swiftly 10μL of each of the protein samples and the Pharmacia Isoelectric Focusing Standards with

TABLE 11. Isoelectric points of the protein standards used for isoelectric focusing

Protein	pI (24°C)
Amylogucosidase	3.50
Soybean trypsin inhibitor	4.55
β-lactoglobulin A	5.20
Carbonic anhydrase B (bovine)	5.85
Carbonic anhydrase B (human)	6.55
Myoglobulin (acidic, horse)	6.85
Myoglobulin (basic, horse)	7.35
Lentil lectin (acidic)	8.15
Lentil lectin (middle)	8.45
lentil lectin (basic)	8.65
Trypsinogen	9.30

a pH range of 3.5-9.3 (Table 11) were applied on the gel.

With the influence of an electric field the ampholines in the gel were able to migrate to a defined pH gradient. The proteins then migrated to their isoelectric points. In order for this to occur, the gel was focused at a constant power of 30W but varying amperage (mA) and voltage (V). Due to the presence of salts in the samples, the gel was watched carefully over the 2h running time. Upon completion, the electrode strips were removed and the gel was fixed for 30 minutes, destained for 5 minutes, stained at 60°C in an air oven for 20 minutes, destained for 24h, and preserved for 1h. The composition of each solution is illustrated in Table 12. The gel was allowed to dry at room temperature for 30-45 minutes. For convenient storage and preserving of the gel, a plastic sheet was then carefully placed over the sticky gel surface. Four days after the run the gels were photographed using Kodak Ektachrome slide film (160 tungsten).

A calibration curve (Appendix F) was established by plotting the migration distance from the cathode and the isoelectric points of the standards. The isoelectric range of the protein sample was determined by extrapolating the distance each band moved from the cathode.

3. Purity Assessment with Straight PAGE

Conventional polyacrylamide gel electrophoresis (PAGE) was used to assess the purity of the major canola protein as described by Fehrnström and Moberg (1977). A LKB 2117 Multiphor apparatus along with a LKB 2103 constant power supply and a Haake Circulating water bath set at 10°C were used. The proportions of the components used to prepare 7.5% and 5% polyacrylamide gels used in conventional PAGE are indicated in Table 13.

TABLE 12. Composition and application time of the isoelectric focusing solutions for detection of bands following electrophoresis

Solution	Time	Solution composition	
Fixative	30 min.	57.5g	trichloroacetic acid (Fisher Chem. Co.,)
		17.25g	sulphosalicyclic acid (Fisher Chem Co.,) and adjust to 500mL with distilled water.
Destaining	a. 5 min. b. 24-36h	500mL 160mL	ethanol, acetic acid, and adjust to 2000mL with distilled water.
Stain	20 min. at	0.46g	Coomassie Brilliant Blue R-250 (Sigma Chem Co.,)
		400mL	destaining solution, Mix and filter through Whatman No.1 filter paper.
Preserving	1h	300mL	destaining solution and 40mL glycerol.

TABLE 13. Composition of 5% and 7.5% Polyacrylamide gels used in straight PAGE

Solution	5% gel conc. (mL of solution)	7.5% gel conc. (mL of solution)
Distilled water	14.9	7.5
Tris-glycine buffer	33.0	33.0
Acrylamide solution	14.8	22.2
Ammonium persulfate solution (1.5%)	3.2	3.2
N,N,N,N tetramethyl ethylene diamine (TEMED)	0.1	0.1

TABLE 14. Stock solutions used in straight PAGE

Solution	Solution Composition
Tris-Glycine Buffer (pH 8.9)	30.04g glycine, 1g sodium azide, adjust pH to 8.9 and adjust the volume to 2000mL with distilled water.
Electrode Buffer	1 part tris-glycine buffer and 1 part distilled water.
0.25% Bromophenol Blue	2.5mg bromphenol blue added to 1mL samples
Acrylamide Solution	22.2g acrylamide (Eastman Kodak Co., Enzyme Grade), 0.6g Bisacrylamide, adjust to 100mL with distilled water and filter through Whatman No. 1 filter paper. Refrigerate in a dark bottle.
Ammonium Persulfate Solution	0.15g ammonium persulfate diluted to 10mL with distilled water. Prepared fresh daily.
Detergent	0.1mL Triton-X diluted to 100mL with distilled water

To each tank in the electrophoretic apparatus, 1L of the electrode buffer (Table 14) was added, then five electrode wicks, soaked in the electrode buffer, were placed on top of the gel on either side of the cooling plate. The system was pre-electrophoresed for 10 minutes at 20mA, then the BioRad Gel Filtration Standards (Table 7) and protein samples of 10μL were applied to the slots on the 5% and 7.5% conventional PAGE gels. After the concentrating step, the field strength was set according to the gel concentration. When the gel concentration was 7.5%, the current was set at 40mA for 2.8h while for the 5% gel, the current was set at 45mA for 2.0h. The gels were then fixed for 30 minutes, stained for 1h, destained for 24h, and preserved for 1h in the buffer solutions indicated in Table 15, wrapped in Bio Wrap (Bio Design Inc., New York) and photographed.

Samples for analysis on conventional PAGE were obtained from the gel filtration columns. A number of chromatography runs were performed and the protein fractions were accumulated from the 0.5M and 2.0M NaCl Sephacryl S-300 gel filtration columns. The first electrophoretic analysis was performed employing samples from the typical absorption curve (Figures 3a and b). Both the shoulder (A) and main peak (B) were pooled separately, then the samples were applied to the PAGE gel with protein concentrations of 2mg/mL. A second run was performed utilising the main peak (B) (Figure 3a and b) obtained from the 0.5M and 2.0M NaCl Sephadex S-300 gel filtration columns to determine if these peaks contained the same molecular weight protein. These samples were also dialysed in either 0.5M or 2.0M NaCl solutions, to see if any dissociation of the protein would occur, and then run on both 5% and 7.5% polyacrylamide gels.

TABLE 15. Composition and application time of the straight PAGE and SDS-PAGE staining solutions

Solution	Time		Solution Composition
Fixative	0.5h[a]	57.0g	trichloracetic acid,
		17.0g	sulphosalicyclic acid,
		150mL	methanol,
		350mL	distilled water.
Stain	1h[a]	1.25g	Coomassie Brilliant Blue R-250 (Sigma Chem. Co.),
		227mL	distilled water,
		46mL	glacial acetic acid.
			Mix and filter through Whatman No. 1 filter paper.
Destaining	24-36h with frequent changes	1500mL	ethanol,
		500mL	acetic acid,
		3000mL	distilled water.
Preserving	1h	300mL	ethanol,
		100mL	acetic acid,
		100mL	glycerol,
		500mL	distilled water.

[a] Time used in straight PAGE should be doubled when preforming SDS-PAGE

4. Subunit Analysis with SDS-PAGE

The molecular weight of the major canola protein subunits were determined according to the sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) procedure described by Fehrnström and Moberg(1977). The stock solutions used during SDS-PAGE are illustrated in Table 16. To provide good thermal contact with the gel plate a few drops of Triton-X 100 were placed between the cooling and the gel plates. The component composition of the 7.5% SDS polyacrylamide gels used are represented in Table 17. One litre of the electrode buffer (Table 16) was placed in each of the tanks of the LKB 2117 Multiphor apparatus. After soaking in the electrode buffer, five electrode wicks were placed on top of the gel on either side of the cooling plate. Once the Multiphor apparatus along with a LKB 2103 constant power supply and a Haake circulating water bath set at 5°C was set up, the system pre-electrophoresed for 30 minutes at 150mA. Then 10μL of each of the protein samples along with the Pharmacia SDS-PAGE Molecular Weight Standards (Table 18) were pipetted into the appropriate gel slot. Electrophoresis was carried out at 10mA for 10 minutes and then increased to 195mA for 4.5h. The gels were then fixed, stained, destained and preserved in the respective buffers described in Table 15 and photographed using Kodak Ektachrome slide film (160 tungsten). For the SDS-PAGE gels, fixing and staining times were double those in Table 15 (respectively 1h and 2h).

From the gel, a calibration curve (Appendix G) relating the relative mobility to the $\log_{10}$ molecular weight of the standards. The subunit $\log_{10}$ molecular weight was determined by extrapolating the distance each band migrated. To prepare the samples for SDS-PAGE, the protein was

TABLE 16. Stock solution used for SDS-PAGE

Solution	Solution composition
Stock Phosphate Buffer (0.2M pH 7.1)	15.6g $NaH_2PO_4 \cdot H_2O$, 40.92g Na_2HPO_4, 4.0g SDS, adjust the pH to 7.1 with 0.2M NaOH, and adjust the volume to 2000mL with distilled water.
Phosphate Sample Buffer (0.01M, pH 7.1)	50μL 2-mercaptoethanol, 50g SDS, 0.15mg bromophenol blue, 50mg urea, and 5mL of protein sample or protein standard solution.
Electrode Buffer	1 part stock buffer and 1 part distilled water.
Acrylamide Solution	22.2g acrylamide (Eastman Kodak Co., Enzyme Grade), 0.6g Bisacrylamide, adjust to 100mL with distilled water and filter through Whatman No. 1 filter paper. Refrigerate in a dark bottle.
Ammonium Persulfate Solution	0.15g ammonium persulfate diluted to 10mL with distilled water. Prepared fresh daily.
Detergent	0.1mL Triton-X diluted to 100mL with distilled water.

TABLE 17. Composition of 7.5% polyacrylamide gels for SDS-PAGE

Solution	Volume (mL) of solution required for a final acrylamide concentration of 7.5%
Distilled water	7.5
Stock Phosphate Buffer (0.2M pH 7.1)	33.0
Acrylamide solution	22.2
Ammonium persulfate	3.2
N,N,N,N Tetramethyl ethylene diamine (TEMED)	0.1

TABLE 18. Molecular weight of Pharmacia SDS-PAGE standards

Protein	Molecular Weight	Log_{10} Molecular Weight
Bovine serum albumin	66,200	4.821
Ovalbumin	45,000	4.653
Pepsin	34,700	4.540
Trypsinogen	24,000	4.380
β-lactoglobulin	18,400	4.265

dissociated into its individual subunits. In the presence of SDS, multisubunit proteins dissociate. Since the major canola protein contains disulfide bonds, a reducing agent (2-mercaptoethanol) must be used to cleave the bonds. By exposing the protein samples to SDS alone, and a mixture of SDS and mercaptoethanol, the molecular weight of the constituent subunits could be determined. In preparing the standards, 1% SDS (w/v), 1% 2-mercaptoethanol and 1% bromophenol blue were incorporated. To all samples containing 0.5 and 1mg/mL protein, 1% SDS (w/v) was added while only half of the samples were reduced with 1% (w/v) 2-mercaptoethanol (Aldrick Chem. Co.). The samples were then incubated at 37°C for 3h in an air oven prior to application to the SDS gel.

5. Determination of Amino Acid Composition

The amino acid content was determined for the dried defatted canola meal and the main canola protein (B) (Figure 3a) isolated by gel filtration using 0.5M NaCl with 0.02% sodium azide as the elution buffer. To remove the salt, the liquid fraction was dialysed extensively against water at 5°C. After dialysis, the protein content was determined by the Coomassie procedure (Pierce Chem. Co., 1986).

a. Hydrolysis of Protein. A sample containing a known amount of protein (1-10μg) was placed into a sample tube (50 x 60mm) which was in turn positioned into the reaction vial. The Waters PICO•TAG work station was used for hydrolysis and precolumn derivatization of the sample for amino acid analysis. Using the vacuum pump on the work station, the samples were properly evaporated when the vacuum gauge reached at least 65 millitorr. After drying the samples, 6N HCl (1mL) with 1% phenol (10μL)

by volume was carefully placed into the bottom of the reaction vial. Hydrolysis was carried out in the PICO•TAG oven at 105-112°C for 20-24h at a pressure of 1-2torr. To redry the sample, a 2:2:1 mixture of ethanol:water:triethylamine was added to each sample tube and vortexed. The tubes were placed in the reaction vial and evaporated on the work station.

Derivatization of the protein samples were carried out by adding 20μL of the freshly prepared derivatization reagent [7:1:1:1 ethanol: triethylamine:water:phenlisothiocyanate (PITC)]. After standing at room temperature for 20 minutes, the reaction vial was evaporated to remove PITC for 45 minutes or until the vacuum gauge reached 65 millitorr.

b. Standard Preparation. Approximately 5μL of the Pierce amino acid standard H (No 20088) was placed in the sample vials and dried on the PICO•TAG work station. The standards were then redried and derivatized using the procedures outlined for the protein sample.

c. Amino Acid Analysis by Reverse Phase HPLC. The above protein samples and standards were assayed for amino acid composition on a Waters Millipore high performance liquid chromatographic (HPLC) system. The system consisted of two Waters pumps; a model 45 for aqueous solvent and Model 510 for organic solvent, a Waters automated gradient controller; model 680, a fixed wavelength (280nm) ultraviolet (U.V.) detector; model 441 and a Hewlett Packard 3390A integrator. The PICO•TAG amino acid analysis column (3.9mm x 1.5cm) was maintained at 38 ± 1°C by a Waters temperature control module (TCM). For sample injection, an U6K manual injector was used. The necessary reagents were purchased as the PICO•Tag

chemistry package.

d. Determination of Specific Amino Acids. After the amino acid content was determined, further steps were required to determine the amount of cysteine and tryptophan in the protein. The cysteine content was determined by methodology described by Pieniazek *et al.* (1975). The procedure identified by Messineo and Musarra (1972) was utilized to assay the tryptophan content in the defatted canola meal.

e. Parameters Estimated from Amino Acid Composition. Directly from the amino acid data, a number of parameters can be estimated to provide general information about some of the physicochemical properties of canola meal and the 12S globulin. The average hydrophobicity ($H\phi_{ave}$), charge potentials (positive, negative and total) and charge frequencies were calculated according to Bigelow (1967). The methodology of Rowe (1978) was used to calculated partial specific volume, while the nitrogen to protein conversion factor was estimated as described by Tkachuk (1969). The equation reported by Alsmeyer *et al.* (1974) were used to predict the protein efficiency ratio from the amino acid analysis.

F. The Influence of Environmental Manipulation on Protein Conformation and Micelle Response

1. Environmental Manipulation of the Major Canola Protein

To establish the influence of pH and ionic strength on the micelle response, the isolated storage protein was dialysed with the salt solutions listed in Table 19. The major storage canola proteins isolated from the 0.5M gel filtration column, as previously described, was concentrated in an Amicon ultrafiltration cell with a molecular weight cut

off of 10,000. The protein solution was concentrated approximately 5-8 times. For each environment, approximately 12mL of the isolated protein was placed in a 15mL glass vial. The pH of the protein solution was adjusted to the pH corresponding to the appropriate buffer with 0.1N NaOH or 0.1N HCl. The protein solution was then placed in a 15-20cm strip of Spectrapor/Por4 cellulose dialysis tubing (16mm diameter with a molecular weight cut-off of 12,000-14,000). The dialysis tubing, with the sample, was then placed in a 500mL beaker and buffer was added. Dialysis continued over a 36-48h period at 4-7°C with the buffer being continuously stirred and changed at least 6 times during dialysis.

After dialysis, the protein concentration was determined as previously described where the environmentally manipulated protein sample was placed into a Minicon B-15 Macrosolute concentrator with a molecular weight cut-off of 15,000 and concentrated to give a final concentration of either 50mg/mL for microscopy work and 100mg/mL for DSC analysis. These final concentrations were confirmed by again determining the concentration of the soluble protein. For samples with greater protein concentrations, the samples were diluted with the appropriate environmental buffer to the desired concentration. The above procedure was duplicated for each salts solution in Table 19 prior to light microscopy and DSC analysis of the sample.

2. Protein Conformational Changes

a. DSC Analysis. The conformational changes of the major canola protein in different environments were monitored by studying the thermal properties of the protein using differential scanning calorimetry (DSC). After the protein was dialysed in its prospective buffer (Table 19), the

TABLE 19. The salt solutions prepared in 0.01M phosphate buffers used to environmentally manipulate protein conformation and micelle response

0.1M NaCl pH 6.0
0.1M NaCl pH 7.0
0.1M NaCl pH 8.0
0.3M NaCl pH 6.0
0.3M NaCl pH 7.0
0.3M NaCl pH 8.0
0.5M NaCl pH 4.0
0.5M NaCl pH 6.0
0.5M NaCl pH 7.0
0.5M NaCl pH 8.0

thermal properties were assessed by a DuPont 990 Thermal Analyzer with a 910 Differential Scanning Calorimeter cell base. On a Mettler AE163 analytical balance, samples weighing 10-15mg were and hermetically sealed in DuPont aluminum pans, coated on the interior with an inert polymer. To improve contact between pans and thermocouple detectors, a silicon heat-sink compound (Dow Corning Corp.) was used. Both the reference, a sealed empty pan, and the sample pan were placed in the DSC cell under nitrogen at a constant pressure of 300psi. The heating rate of the sample was 10°C/minute over a temperature range of 25°C to 130°C. After each run, the cell was cooled to room temperature with ice. Duplicate samples were analyzed at least in duplicate.

To collect and analyze the thermal data the DSC Standard Data Analysis Program (Version 2.2C) was used. The results were graphically illustrated as a thermal curve where heat flow was a function of temperature. From the thermal curve, the temperature of maximum heat flow into the sample or thermal denaturation temperature (Td) and the area of the endothermic thermal curve or enthalpy of denaturation (ΔH) were determined. After analyzing the thermal properties of the protein, the thermal curve was plotted by a Hewlett Packard model HP7470A graphics plotter.

b. Assessment of the Micelle Response. A Zeiss Universal Research light microscope was used to assess the effect of environmental manipulation, protein concentration and dilution on micelle formation and self-association. Nine different environments, with varying ionic strength and pH, indicated in Table 19 were used to manipulate the protein conformation and micelle response. In each specific environment, the effect of protein

concentration (50, 25 and 10mg/mL) on the micelle response was observed. Within each protein concentration level, 5μL protein samples were diluted with ice cold water to ratios of 1:5, 1:10, 1:15, 1:20, 1:25, and 1:30. Each experimental condition was duplicated with two different protein samples from the same environment.

Due to the amount of time, preparation and chemicals required to produce the final environmentally manipulated protein, samples with very small volumes (5μL) were examined under the microscope. The ratio of salt, protein and water were believed to be very important, so were carefully controlled to ensure reproducibility.

Protein samples of 5μL were pipetted onto clean glass slides. The slide was then observed prior to dilution under the Zeiss light microscope. Photographs were taken of any particulate matter or insoluble protein observed in the protein sample. The sample at room temperature was then diluted with distilled water at 4-7°C and a coverslip was carefully placed over the diluted protein solution. The slide was observed under the microscope and photographs of representative structures for each experimental condition were taken. While observing the response, careful attention was directed towards the size, homogeneity and distribution of the micelles. Micelle aggregates were assessed for size, number and distribution of the aggregates as well as the size of constituent micelles within the aggregates. Mixtures of aggregates and individual micelles were also assessed. The optimum or desired response was the formation of networks and protein sheets. When formed, the size of the integral micelles, the texture and continuity of the network or sheet were noted.

In an attempt to obtain concrete numbers pertaining to the micelle response an Olympus BH2 microscope was connected to an IBM T/S2 computer equipped with the Q2CUE-2 Image Analyzer program, which generates numbers primarily of individual of clusters of objects was used. The high light microscopy magnification required to view some micelles and the dense nature of the desired networks prevented effective utilization of the image analyze to suit the needs of this research. The numbers or results generated from the image analyzer failed to provide representative information or numbers for aggregation and network areas.

IV. RESULTS AND DISCUSSIONS

A. Proximate Analysis

The proximate analysis results presented in Table 20 indicated the Westar canola meal contained 9.95% moisture, 4.40% fat and 35.6% protein (N X 5.67). Correspondingly, these values were comparable to values for the Westar meals analyzed at CSP Foods for the 1990 season: 10% moisture, 5% fat and 38% (N X 6.25) (Personal communications with Dave Thiessen). Small protein variations could be attributed to different nitrogen conversion factors that were used to determine the percentage of protein. The ash content of rapeseed in the literature varied with the species or cultivar of the sample. Appelqvist and Ohlson (1972) found the ash content ranged from 7.0% to 7.5% while Bell (1989) observed lower ash values of 6.3%. Thus, the experimental value of 6.94% was within the range of percentages observed in the literature. According to Ohlson (1985), the phytic acid content, on a dry weight basis, for rapeseed can vary from 1.5 to 2.4% while Welsh observed a value of 2.00% with canola. The experimental value for the phytic acid content (1.63%) (Table 10) was comparable and within the range reported in the literature. In this study, the phenolic content was 0.028% which was similar to the 0.034% value obtained by Welsh (1988) from raw defatted canola meal.

B. Isolation of Canola Protein by Micellar Mass Procedure

Welsh (1988) used the PMM procedure to isolate canola protein at

TABLE 20. Proximate analysis of starting material

COMPONENT	% (DRY WEIGHT BASIS)
MOISTURE	9.95±0.04
PROTEIN	35.6±1.7
LIPID	4.40±0.23
ASH	6.94±0.02
PHENOLIC	0.0278±0.002
PHYTIC ACID	1.63±0.04

three different pH values: 5.5, 6.0 and 6.5 (Table 21). Within this range very low protein recoveries were obtained and Welsh (1988) demonstrated that pH manipulation did not significantly vary the amount of protein isolated. As a result, slight modifications were made to the PMM procedure used by Welsh (1988) to increase protein recoveries, thereby increasing the feasibility of the PMM procedure for commercial use. These modifications included an increase in the amount of meal used in the extraction procedure was increased from 25g to 200g and 500g. This increase in original meal was performed to minimize losses due to sample manipulation and to give an indication of the effect of scaling up. By increasing the meal approximately 10 times and 20 times, the protein yields increased from 6.2% to 20.9±1.9% and 31.0±5.5%, respectively.

Using the procedure as described by Welsh (1988), some of the pellet remained interdispersed in the supernatant. After centrifugation, recovering the high salt protein extract was very difficult, as the supernatant required further filtration through cheesecloth where some of it was absorbed. To overcome this problem, a second modification was introduced using higher centrifugation speeds. Welsh (1988) used a Sorvall Refrigerated Centrifuge, Model RC2-B, at 16,000xg for 30 minutes which was altered by using a Sorvall Refrigerated Centrifuge, Model RC-3, at 150,000xg for 30 minutes. This modification compressed the cellular debris at the bottom of the 1000mL centrifuge bottles where the supernatant was easily decanted off. Filtering through cheesecloth was unnecessary and sample losses were minimized.

Gillberg and Törnell (1976a) indicated the effect of increasing the speed of centrifugation from 500 to 3,000 RCF did not influence the amount

TABLE 21. Recoveries of protein from defatted canola meal using the protein micellar mass procedure

pH 5.5		pH 6.0		pH 6.5	
0.01M NaCl[2]	**0.1M NaCl[3]**	**0.01M NaCl[2]**	**0.1M NaCl[3]**	**0.01M NaCl[2]**	**0.1M NaCl[3]**
1.35±0.62[a]	3.85±0.57[b]	1.96±0.20[a]	3.86±0.62[b]	1.46±0.42[a]	4.29±0.73[b]

[1] Each extraction was carried out using a 25g of raw canola meal.
[2] All buffers were prepared with 0.01M NaCl in 0.01M Na_2HPO_4.
[3] All buffers were prepared with 0.1M NaCl in 0.1M Na_2HPO_4.

Values followed by the same letter are not significantly different at 0.05 level.

Welsh (1988)

of total nitrogen extracted, but decreased the phytic acid content in the extract. A decrease in the extract turbidity was also observed with increasing centrifugal force which paralleled the decrease in phytic acid solubility. These results implied that the turbidity of the extract was related to the presence of finely divided insoluble phytic acid derivatives. Thus, increasing the centrifugal force to effectively separate the cellular debris and the supernatant should also beneficially reduce the amount of phytic acid in the recovered supernatant.

A third modification to the PMM procedure was introduced by concentrating the supernatant in a H1P10-20 Diaflo Hollow Fiber Concentrator instead of an Amicon ultrafiltration cell with a 180mL volume capacity. The molecular weight cut-off of 10^4 remained the same in both systems. The use of the hollow fiber concentrator allowed larger volumes of supernatant to be rapidly concentrated and eliminated handling losses that occurred during the refilling of the Amicon cell. With this system, the supernatant was reduced by at least eight times its original volume.

Although not investigated in this study, the molecular weight cut-off of the ultrafiltration membrane can influence protein recovery and removal of non protein material. Comparing the molecular weight cut-offs of 5,000, 10,000, 50,000 and 100,000 for various Hollow Fiber membranes, Diosday and associates (1984) indicated the most effective removal of impurities with minimal loss of rapeseed protein (4.9%) was obtained with the 50,000 molecular weight cut-off membrane. Interestingly, due to concentration polarization, the 50K membrane demonstrated reasonably good retention of proteins with molecular weights less than 50,000 daltons. With the 100K membrane, protein loss was large while the impurity removal

of the 5K and 10K membranes was not as efficient as the 5K membrane.

Although the removal of impurities was best with the 50K membrane, high levels of some contaminating factors can be removed regardless of the membrane size. This impurity removal is an added benefit to the concentration step. Glucosinolates, for example, have a low molecular weight (390-470 daltons) and readily permeate through the membrane independent of the membrane pore size. Diosady *et al.*(1984) reported retention values of about 8.2% for glucosinolates. The phytate content has also been reduced from 128mg phytic acid/g protein to 18-19mg phytic acid/g protein through the use of hollow fiber ultrafiltration system (Kroll *et al.*, 1989).

In the present research, only a 10K membrane was used, in order to compare the effectiveness of the hollow fiber system to the ultrafiltration membrane used by Welsh (1988). Also, the increased sample sizes used necessitated a more efficient concentrating system. The increased yields were evidence of the effectiveness of these modifications. The use of membranes with higher molecular weight cut-offs should only improve this procedure, yet it is recommended that nothing higher than 50,000 be used as the smallest dissociation product (2-3S subunit) has a molecular weight of 50,000.

By incorporating these three modifications changes to the PMM procedure, the overall recoveries were increased from approximately 4.00±0.25% (the average of Welsh's (1988) recoveries with 0.1M NaCl in 0.1M Na_2HPO_4) to 20.93±1.92% with 200g of starting material and 30.97±5.51% with 500g of starting material. Although these recovery values were still low, they provide a good indication that protein recoveries should

increase when scaling up the PMM procedure to pilot plant scale.

In the past, the PMM procedure has been utilized to isolate fababean (Ismond *et al.*, 1984; Georgiou, 1987), safflower (Paredes-Lopez *et al.*, 1988) and oat protein (Ma, 1983; and Ma *et al.*, 1990). For seed proteins, the PMM procedure has been shown to be advantageous because it was a simple and mild technique which selectively isolates globular proteins with residual amounts of contaminating factors. When comparing isoelectric protein isolates (IPI) with micellization protein isolates (MPI), Paredes-Lopez *et al.*, (1988) demonstrated lower protein yields were observed with the PMM procedure. However, the MPI were less denatured, and had better color, solubilities and functional properties than IPI. Ma (1983) compared the composition and functional properties of oat protein isolates prepared by the PMM procedure and isoelectric precipitation. Both isolates contained 90% protein, however, the yield was much higher in the IPI (60%) than the MPI (25%). Ma (1983) postulated this difference occurred as the PMM procedure extracted only the globulin protein while the isoelectric process precipitated globulins and the other proteins in the Osborne fractionation scheme. Since the PMM procedure selectively isolated globulin, which are quantitatively low in oats, low recoveries were obtained.

The PMM procedure appears to be very uncomplicated, however, the methodology must be manipulated to accommodate the physicochemical properties of the specific protein (Table 2). Each different plant protein requires a unique salt or salt concentration to extract the protein as well as a distinctive solvent pH and dilution factor. To optimize the amount of protein retrieved and reduce the antinutritional

factors several parameters must be controlled including:

-initial salt concentration;
-pH of the solubilizing medium;
-protein concentration in the salt extract;
-dilution factor.

In evaluating the isolation procedure for recovering proteins these factors must be considered:

-amount of antinutritional factors (glucosinolates, phytic acid and phenolics);
-protein content of the isolate;
-protein recovery;
-protein quality and stability;
-protein functionality.

With these guidelines in mind, the protein isolates acquired by Welsh (1988) were evaluated. The canola isolate contained residual amounts of antinutritional factors consisting of 0.61% glucosinolates; 0.49% phytic acid and 0.005% phenolics. Under optimum extraction conditions 0.1M NaCl in 0.1M Na_2HPO_4 at pH 5.5, Welsh (1988) isolated a product containing 80.78% protein. In addition, with slight procedural modifications, such as larger sample sizes, protein recoveries could be increased above the 2 to 5% obtained by Welsh (1988). The assessment of protein quality or stability can be determined by evaluating the thermal properties using DSC. Welsh (1988) reported a thermal denaturation temperature (Td) or the temperature at maximum heat flow, of 87.87°C, and an enthalpy of denaturation (ΔH) or the heat required to unfold the protein, of 5.69 J/g and suggested that this represented a fairly native isolate. Yet, in the present study, the Td value varied from 82 to 93° in various environments. At pH 6.0, the Td value was 83°C which was significantly lower than in the 0.3M NaCl pH 6.0 (89.43±0.05) or 0.5M NaCl pH 6.0 (91.43±0.03) (Appendix L). Thus, in the 0.1M environment, the

protein appeared to be less stable. In present research, the average ΔH value was roughly 24 J/g (Appendix L) which was notably larger than a ΔH value of 5.69 J/g observed by Welsh (1988) but similar to a value of 24.06 J/g protein (Murray *et al.*, 1985) was reported previously for a canola micelle isolate prepared from canola. Consequently, the structural integrity of the extracted protein in the 0.1M NaCl pH 5.5 environment used by Welsh (1988) seems questionable.

Possibly, one of the factors contributing to the poor protein recoveries and the proteins structural integrity was the utilization of low ionic strength buffers (0.01M and 0.1M NaCl). In the literature, a number of researchers have indicated the use of high NaCl concentrations (5-10% or respectively 0.86M-1.71M) resulted in high protein extractabilities of 66-98% (Mieth *et al.*, 1983b). Evidently, by increasing the molar concentrations of the NaCl used in the extract from 0.1M to 0.5M or 2.0M, the amount of soluble protein in the extract should increase and ultimately increase the protein recoveries of the PMM procedure. In addition, Schwenke *et al.* (1983) indicated the 12S could dissociate to 7S globulin in low ionic strength solutions. The ionic strength of the 0.1M environments could be low enough to dissociate the protein, thus explaining the low ΔH values observed by Welsh (1988).

C. Solubility Assessment of Canola Meal

1. Effect of NaCl Concentration on Protein Extractability of Canola Meal

The above concerns about poor protein recoveries and structural conformation, promoted a solubility study on the canola meal. An objective of this research was to optimize the extraction of canola

globulin proteins by observing the effect of salt concentration, time, and the meal to solvent ratio on protein extractability from the defatted meal. The results of this solubility study are indicated in Appendix H. It was demonstrated that increasing the meal to solvent ratios from 1:10 to 1:20 did not result in increased nitrogen extractabilities. Therefore, the ratio was set at 1:10 for subsequent runs. When considering the effect of time upon nitrogen solubility, extractions were performed at 1h, 2h and 4h intervals. The 4h extractions solubilized significantly more protein than 1h, however only slightly more than the 2h. As a result, 2h extractions were used to determine the effect of NaCl concentration on nitrogen extractabilities in further trials (Appendix H and Figure 4) as the benefit achieved through the additional time was insignificant.

The effect of NaCl concentration on nitrogen extractibility is presented in Figure 4 which indicated that increasing the NaCl concentration from 0.05M to 0.5M elevated protein recoveries. Above 0.5M NaCl, the protein recovery seemed to plateau (or decrease slightly) and then increase again with 2.0M NaCl, such that the recovery at 2.0M NaCl was significantly higher than with 0.75M, 1.00M and 1.50M NaCl. It should be noted the value at 2.0M was not significantly different from that at 0.5M NaCl. As a result, two maximum points of nitrogen solubility were observed at 0.5M NaCl (47.03±0.83) and 2.0M NaCl (52.19±1.04). Initially, the two maximum points were postulated to be related to the isolation of both the 7S and 12S globulins. However, the overall low solubilities could be partially attributed to handling losses during filtering. Since the extractions were performed on a very small scale (5g of starting material), the extracts were centrifuged using a Sorvall Refrigerated

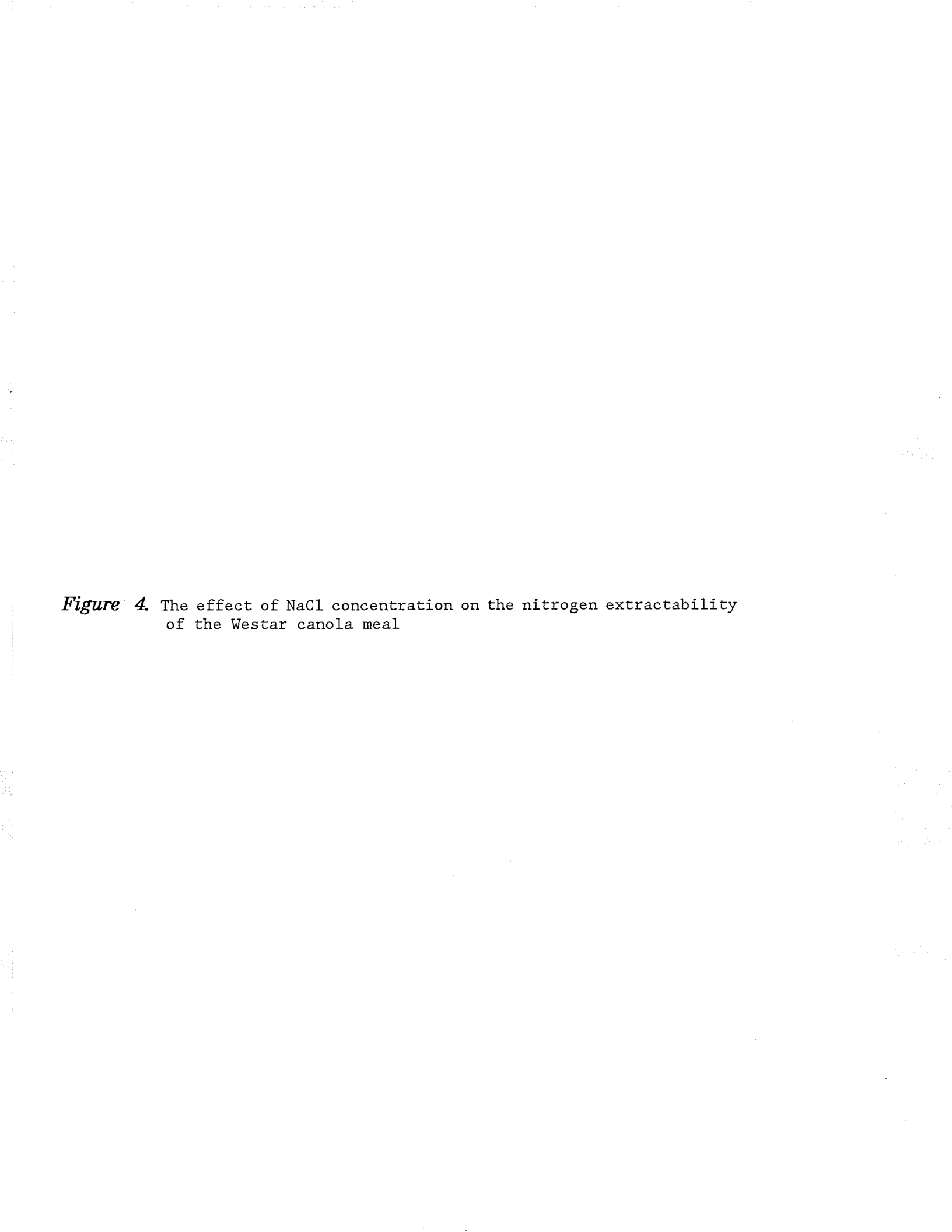
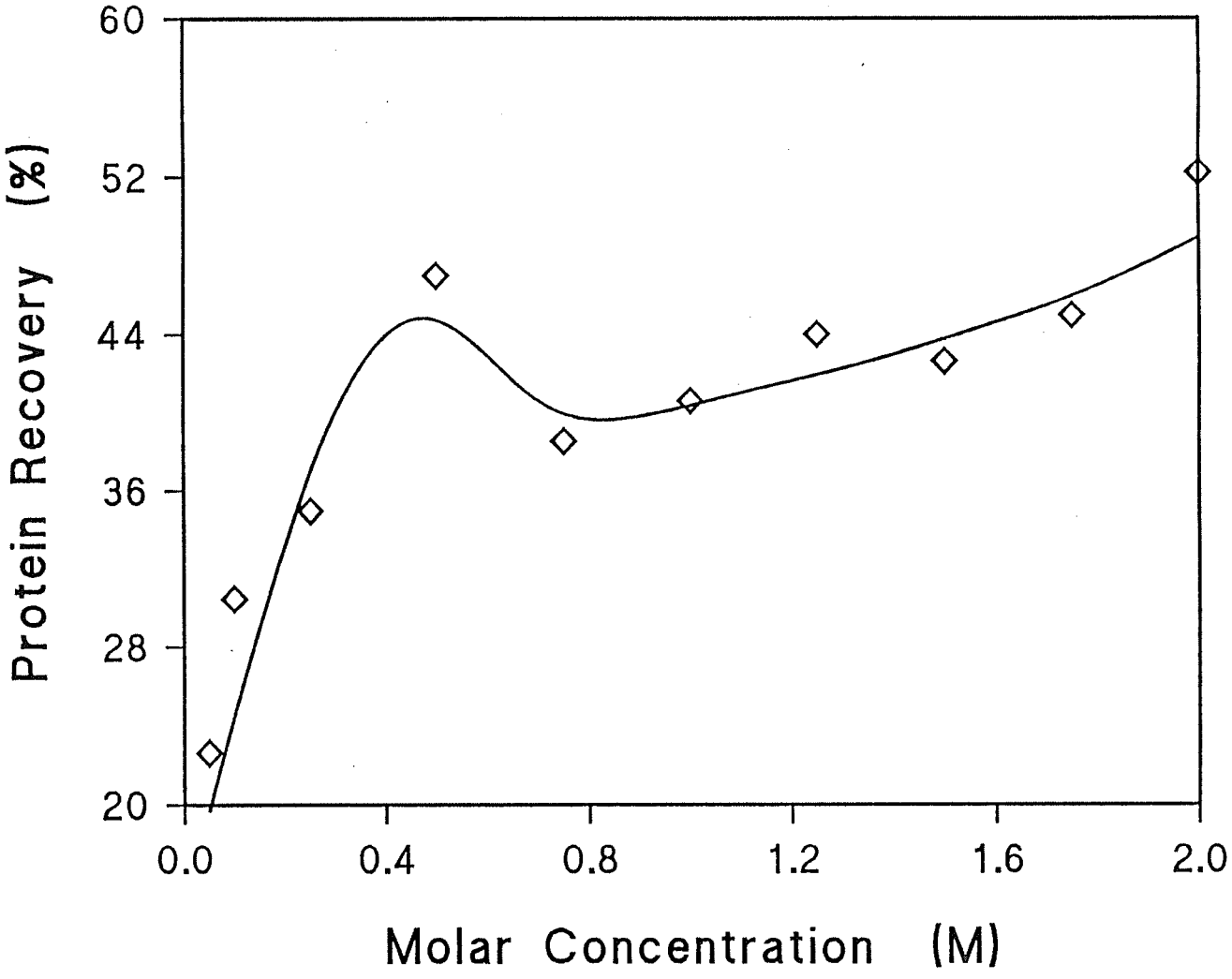


Figure 4. The effect of NaCl concentration on the nitrogen extractability of the Westar canola meal


60
52
44
36
28
20
Protein Recovery (%)
0.0
0.4
0.8
1.2
1.6
2.0
Molar Concentration (M)

Centrifuge, Model RC2-B, at 16,000xg for 30 minutes. As a result, the cellular pellet was very soft and readily dispersed within the supernatant. To separate the salt soluble protein from the cellular debris, filtering through four layers of cheesecloth and Whatman No. 4 filter paper was required. This filtering resulted in large supernatant losses which ultimately created deviations within replicate recoveries. Consequently, the high handling losses were identified as creating artificially low extraction values throughout the study and high standard deviations for most environments.

The effect of pH on protein solubility was not investigated, however, typical pH values of the unadjusted extract ranged from 5.9 to 6.0 (Appendix I). Welsh (1988) identified a pH range of 5.5 to 6.5 did not alter the protein recoveries of the PMM procedure (Table 21). Since these pH solutions were used to extract the protein from the meal, a change in pH over this range was expected to have little effect on protein solubility. Since pH extremes can adversely effect protein conformation, future studies could investigate the effect of pH on protein solubility.

2. Effect of Sodium Hexametaphosphate Concentration on Protein Extractability of Canola Meal

A recent trend in the literature was to extract canola proteins with polyphosphate complexing agents (Bhatty *et al.* 1968; Thompson *et al.* 1976; Thompson *et al.* 1982a; 1982b; Tzeng *et al.* 1988). Thompson *et al.* (1976) examined the effect of the meal to solvent ratio, time and % SHMP concentrations (Table 22) on the extraction of nitrogen from rapeseed flour at pH 7.0. They concluded a ratio of 1:10 was best for nitrogen

TABLE 22. The Effect of Various Processing Parameters on the Extraction of Nitrogen from Rapeseed Flour at pH 7.0

Variable	Nitrogen Extraction Yield (%)
Rapeseed flour to solvent ratio[a]	
1:5	80.10
1:10	81.65
1:15	81.57
1:20	81.62
Time[b] (min.)	
30	81.65
60	81.64
90	81.30
120	82.23
SHMP concentration[c] (%)	
0	61.25
0.1	64.75
0.5	71.69
1.0	73.95
1.5	78.16
2.0	81.65
3.0	81.16

[a] 2% SHMP, 30 min., 25°C.
[b] 2% SHMP, 1:10 ratio, 25°C.
[c] 1:10 rapeseed flour to solvent ratio, 30 min, 25°C.

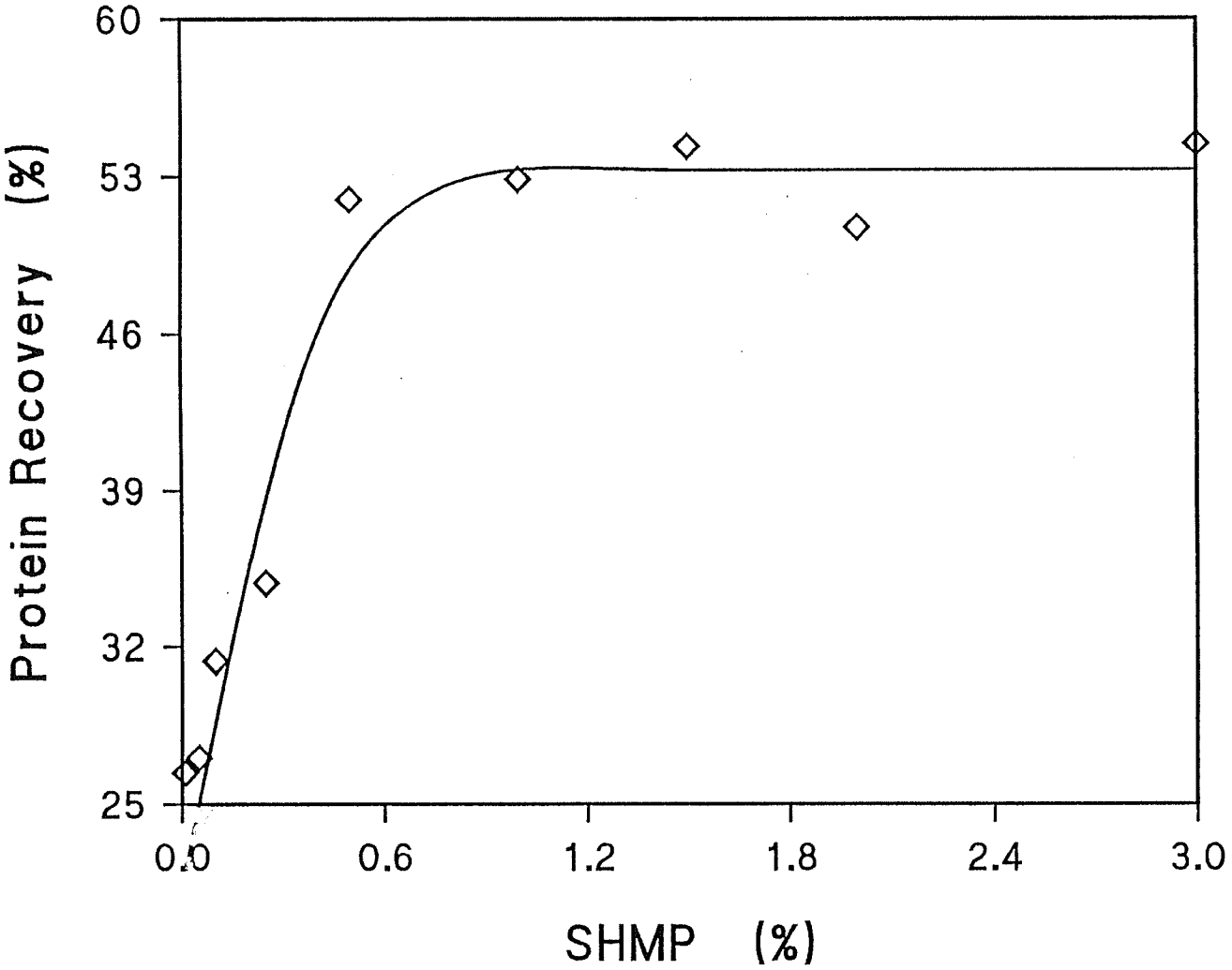
Thompson *et al.* (1976)

extraction, increasing the proportion of solvent had no further effect on the extraction yield, and the time selected for extraction was 30 minutes. For the current experimental procedure, a ratio of 1:10 was used in conjunction with a 2h extraction period. When considering the results of Thompson *et al.* (1976), stirring for the extra 1.5h did not significantly increase the extraction yield, but the two hour extraction coincided with the extraction conditions used in the NaCl solubility study.

The effect of the SHMP concentration (%) on the extraction of nitrogen from canola meal is indicated in Appendix J and Figure 5. In agreement the research of Tzeng *et al.* (1988), the nitrogen extractability increased with increasing SHMP conditions up to 0.5%, then there was no significant change with further SHMP concentrations. However, Thompson and associates (1976) found the extraction yield to be optimum with 2% SHMP. At 2% SHMP their yields of approximately 82% were higher than the 75% obtain by Tzeng *et al.* (1988) or the 52% (Appendix J and Figure 5) received in this experimentation. This deviation may be due to starting materials used as Thompson *et al.* (1976) employed rapeseed flour, while in this experiment and the research of Tzeng *et al.* (1976) raw defatted canola meals were utilized. The protein content of the rapeseed flour used by Thompson *et al.* (1976) was 51.04% (N x 6.25) while the canola meal used by Tzeng *et al.* (1988) contained 39.2% protein (N x 6.25) and the Westar meal in this study contained 35.6% protein (N x 5.67). In addition, the protein solubility of canola meal was further limited by the presence hulls which bind the protein.

In this investigation, only single extraction were used. Consequently, the double extractions used by other researchers (Thompson

Figure 5. The effect of sodium hexametaphosphate concentration on the extractability of nitrogen from Westar canola meal.


60
53
46
39
32
25
Protein Recovery (%)
0.0
0.6
1.2
1.8
2.4
3.0
SHMP (%)

et al. 1976; Thompson *et al.* 1982b; Tzeng *et al.* 1988) elevated the overall yields observed. In the initial extraction, Thompson *et al.* (1976) removed 45-51% of the protein over the examined SHMP range which was comparable to the recoveries in Appendix J at SHMP concentrations of greater than or equal to 0.5%. This research, did not manipulated the pH which ranged from 6.1 to 6.2 within the various extracts (Appendix J). A pH of 6.1-6.2 may have restricted extraction yields, since both, Thompson *et al.* (1982b) and Tzeng *et al.* (1988) established nitrogen solubility escalated with increasing pH. The maximum yield was 90% at pH 12 (Thompson *et al.*, 1976). However, in this range, the extracts were undesirably dark in color, thus, both researchers suggested employing pH 7.0 where extraction yields were nearly 80%. In examining Thompson *et al.* (1976) results, the nitrogen extractability increased by 12% from pH 6.0 to 7.0. Since the present research was carried out at pH 6.1 to 6.2, reduced recoveries were anticipated.

In this study, with the higher SHMP concentrations, the extracts became dark in color and it was possible that the hexametaphosphate anion was complexing with phenolics in the meal. Subsequently, the resulting isolates would also likely be undesirably dark in color. Appelqvist and Ohlson (1972) and Lo and Hill (1972) indicated the phenolic constituents are responsible for green and brown colors of rapeseed products. Thus, further steps would be required to remove these antinutritional factors from the isolate.

Comparing the 0.5% SHMP (Appendix J) and 0.5% NaCl (Appendix I) extractions in this study, the nitrogen solubilities were similar with protein recoveries of 52% and 47%, respectively. Even though there was

potential for extracting more protein with SHMP with slight procedural modifications, further extraction with SHMP were discontinued for many reasons. Firstly, Thompson *et al.* (1982b) and Lui *et al.* (1982) expressed environmental and biological concerns about the use of SHMP to extract rapeseed proteins. In addition, reduced ionic strengths and low pH values (≤2.5) were required to optimally precipitate the protein. When using acidic precipitation at low pH ranges (≤3.0), there was a possibility that the 12S globulin could be irreversibly dissociated to the 2-3S globulin. Furthermore, upon dilution, the protein contained high amounts of phosphorous. For example, when extracting with 2% SHMP, the isolate contained 3.2-3.3% phosphorous (Thompson *et al.* 1976; 1982b). Due to the formation of hexametaphosphate-protein complexes, the ash content of the isolate was double that of the starting flour. This could be reduced by lowering the amount of SHMP used to extract the protein. After performing feeding trials, Thompson *et al.* (1982) indicated that rats developed enlarged kidneys when fed ratios containing 20% of the isolate obtained from 2% SHMP extracts. The combination of these concerns with fact that NaCl is cheap, readily available and an acceptable food ingredient, enhance the attractiveness of NaCl over SHMP. Thus, further SHMP extractions were discontinued.

D. Characterization

Canola meal consists mainly of two physicochemically discrete proteins; the 1.7S albumin and the 12S globulins. Albumins, water soluble proteins, can be easily segregated from the large salt soluble 12S globulins by gel filtration or ion-exchange as they are small (12,000-18,000) and basic (pI>9.0). Salt soluble globulins dissolve in NaCl

solutions where albumin are insoluble. Unlike other plant proteins, the 7S globulin of canola does not appear to be a separate entity or protein, rather it is a dissociation product of the 12S globulin. Schwenke *et al.* (1981) suggested the sensitivity of the 12S globulin to changes in ionic strength and pH could cause it to reversibly dissociate to the 7S trimer (158,000) or irreversibly to the 2-3S subunit (50,000). In addition, an aggregated 17S protein has been identified. Thus, when isolating, purifying or characterizing the protein, the surrounding milieu must be considered to ensure dissociation does not occur.

1. Molecular Weight Determination

a. Gel Filtration. To investigate the possible dissociation of the 12S protein, the molecular weight of the major salt soluble proteins were estimated in both the 0.5M and 2.0M NaCl extracts. Preliminary runs were carried out by applying the 0.5M NaCl extract to the Sephacryl S-300 column equilibrated with 0.5M NaCl solution while the 2.0M NaCl extract was applied to the sephacryl S-300 column equilibrated with 2.0M NaCl solution. Following chromatography, the molecular weight of the main salt soluble fractions were calculated from their respective calibration curves based on proteins of known molecular weights (Appendix A and B). On average, the molecular weight of the main fraction from the Sephacryl S-300 column equilibrated with 0.5M was 253,000±65,000, while on a similar column equilibrated with 2.0M NaCl the molecular weight was 165,000±16,000 (Table 23). These experimental values were lower than the theoretical value of 295,000 for the 12S globulin suggested by Schwenke *et al.* (1983) (Table 2) and indicated the protein in the 2.0M NaCl was smaller than the

TABLE 23. The molecular weight of the major salt soluble protein determined on Sephacryl S-200 and S-300 gel chromatography columns equilibrated by 0.5M and 2.0M NaCl solutions.

Column	Column Buffer (M)	Extraction Condition (M)	Condition Upon Application (M)	Molecular Weight
Sephacryl S-300	0.5	0.5	0.5	253,000±65000
	2.0	2.0	2.0	165,000±16000
Sephacryl S-200	0.5	0.5	0.5	223,000±20000
	2.0	2.0	2.0	200,000± 3600
Sephacryl S-200	0.5	2.0	0.5	223,000± 3000
	2.0	0.5	2.0	202,000± 9000
Sephacryl S-200	0.5	2.0	2.0	200,000± 7800
	2.0	0.5	0.5	250,000±38000

0.5M NaCl. After separately pooling the protein from the 0.5M and 2.0M NaCl Sephacryl S-300 columns, the pooled protein samples were rerun on the 0.5M and 2.0M Sephacryl S-200 columns to further check the molecular weight values. The molecular weights were found to be 223,000±20,000 on the 0.5M column and 200,000±3,600 on the 2.0M NaCl column. To check for dissociation of reassociation with different salt environments, the 0.5M combined protein fractions were dialysed for 36h in 2.0M NaCl and applied to the 2.0M Sephacryl S-200 column, the resulting molecular weight was 202,000±9,000. Similarly, 2.0M pooled fractions were dialysed for 36h in 0.5M NaCl and applied to the 0.5M Sephacryl S-200 column, resulting in a protein fraction with a molecular weight of 223,000±7,300. In other words, no change in the molecular weights were observed when a 0.5M NaCl pooled fraction was directly applied to a 2.0M Sephacryl S-300 column without dialysis, the molecular weight was found to be 250,000±38,000. Conversely, when a 2.0M NaCl fraction was directly applied to the 0.5M Sephacryl S-300 column the molecular weight was calculated to be $2.00 \times 10^5 \pm 7,800$. Generally, the experimentally determined molecular weights (Table 23) ranged between the expected molecular weight value of the 7S (158,000) and the 12S (295,000) globulins with most values at 200,000-250,000.

The maturity of the seed can influence the type of globulin located in the seed. Since the 12S globulin functions as a nitrogen source during germination, it will accumulate as the seed develops under favorable conditions. Norton (1989) indicated that if low amounts of sulfur were present the percentage of 12S developed would be less. Nonetheless, other techniques should be utilized to try and determine the molecular weight of

the purified protein fraction.

In Canada rapeseed / canola breeders have dramatically changed the genetic composition of canola to produce seeds low in glucosinolates and eruric acid. While selecting seeds with low glucosinolates and erucic acid content, breeders may have changed the genetic makeup of the constituent proteins. Consequently, in Canadian canola, the formation of 7S globulin maybe relatively lower or nonexistent compared to that of the European rapeseed used by Schwenke *et al.* (1981; 1983).

In an attempt to explain the low molecular weights of Gill and Tung (1976) (129,000) and Simard *et al.* (1978 as cited by Schwenke *et al.* (1981) (169,000); Schwenke *et al.* (1981) assumed the molecular weight of the 7S trimeric half molecule (158,000) was similar to that of other 11/12S plant proteins. In the research of Gill and Tung (1976), the protein fraction was isolated by alkali precipitation at pH 9.2. Possibly, this extreme pH caused the 12S globulin to dissociate to the 7S globulin with a molecular weight of 129,000. However, the presence of the 7S fraction in *Brassica* species, especially rapeseed, was insinuated to be low or nonexistent by numerous researchers (Bhatty *et al.*, 1968; MacKenzie and Blakely, 1972; Prakash and Rao 1986; Norton 1989). Prakash and Rao (1986) suggested that if present the 7S had a very short half life. According to Schwenke *et al.* (1983) the molecular weight determination or isolation of the 7S product has yet to be performed. As a result, in their research, they were assuming that this molecular weight would correspond to the trimeric half molecule of the native 12S protein,

as in the case of other 11/12S plant globulins. Thus, it seems Schwenke *et al.* (1981; 1983) were generalizing and trying to postulate why low molecular weights were observed by Gill and Tung (1976). This may explain why no dissociation product was observed in canola in this study.

Generally, the molecular weight from the gel filtration runs in Table 26 were below the theoretical 12S molecular weight postulated by Schwenke *et al.* (1981; 1983). According to the theory of Schwenke *et al.* (1981), an ionic strength of 2M and the neutral pH used in this experiment should have inhibited the 12S from dissociating to the 7S. In this research, however, the lowest molecular weight average (165,000±16,000) was observed on the 2.0M NaCl Sephacryl S-300 column. It seems inconceivable that an ionic strength of the 2.0M supernatant was low enough to promote the dissociation of the 12S globulin to the short lived 7S form. Since the molecular weight of the proteins in the 0.5M NaCl solutions were equal to or greater than those in the 2.0M NaCl solutions, it seemed logical to conclude that the 0.5M NaCl extracts also discouraged dissociation. Further reductions in ionic strength may be required to dissociate the protein. Since both the 0.5M and 2.0M NaCl solutions were eluting the same protein, the question arose to why was the molecular weight for the 12S was lower in the high ionic strengths.

In addressing this question, several factors relating to the use of gel filtration for molecular weight determination must be considered. First, the molecular weight range of the matrix used will affect the separation. For example, in previous work with a Sephadex G-100 gel matrix, both, 12S and 17S fractions would elute at the void volume (Bhatty

et al., 1968; Gill and Tung, 1978a) whereas they could be separated on a Sephadex G-200 matrix, (MacKenzie and Blakely, 1972). In this present study, while the broad range for the Sephacryl S-300 matrix ($1x10^4$ to $8x10^6$) should provide better separation of the larger proteins (12S and 17S), while the S-200 matrix ($5x10^3$ to $3x10^5$) should separate the 7S and 12S proteins. Despite this, the volume separating known standards was not great. With thyroglobulin (MW 670,000) the elution volume was 260 mL, while with γ-globulin (MW 158,000) it was 308mL, a difference of only 48mL for a molecular weight difference of 512,000. Thus, the two experimental proteins with molecular weights of 158,000 (7S half molecule) and 295,000 (12S globulin) would be separated by a tube volume of 20mL and any variations in tube volume (a problem which can arise due to salt deposits on the fraction collector) could seriously jeopardize the molecular weight calculation.

In addition, it was noted that when column repacking was required the average molecular weight tended to increase. This increase seemed to be related to compression of the column, a situation which was particularly noticeable with the 2 NaCl column where column height changed by as much as 10-15cm. This increased compression may be responsible for the lower apparent molecular weights with this column.

To overcome this compression problem, a HiLoad 16/60 Superdex 200 prep grade Pharmacia gel filtration column with a molecular weight cut-off range of $1x10^4$ to $6x10^6$ was also used and the respective calibration curves are illustrated in Appendix C and D. The molecular weight range of this column was less than that of the Sephacryl S-300 matrix. So, it was anticipated that the proteins would elute more evenly through out the run.

To more accurately determine the molecular weight of the eluting proteins the column was standardized with thyroglobulin, ferritin, catalase, γ-globulin, bovine serum albumin and ovalbumin. Despite the molecular weight differences of catalase (232,000) and γ-globulin (158,000), the elution volumes were essentially the same. Thus, the volume between proteins was very small and did not allow good peak resolution. As a result, little work was performed using this column because a very narrow elution volume existed in the area of interest. Nevertheless, the elution profiles and molecular weights of the proteins eluted in 0.5M NaCl pH 6.0 and 0.5M NaCl pH 4.0. are given in Figure 6 and Table 24. Interestingly, in the 0.5M NaCl pH 4.0 environment, the protein eluted with a molecular weight of 53,500, indicating the low pH caused the 12S globulin to dissociate to the 2-3S globulin.

Experimentally to this point, the actual molecular weight of the major canola protein was not accurately determined, however, both the 0.5M and 2.0M NaCl extracts solubilized the same protein. The low ionic strengths, which have been insinuated in the literature to cause dissociation of the 12S globulin have not been used in the isolate the protein, but were used later when environmentally manipulating the micelle response. Thus it was doubtful the 12S globulin dissociated during extraction conditions. The ability of the 2.0M NaCl solution to compress the Sephacryl matrix probably artificially lowered the molecular weight values. Alternatively, other techniques should be employed to determine the molecular weight of the purified protein fraction. The next logical technique to try would appear to be ultracentrifugation. The inability of the purified protein to dissolve in the required solutions

Figure 6. Typical elution profiles for the HiLoad 16/60 Superdex 200 Pharmacia gel filtration column
A) equilibrated with 0.5M NaCl pH 6.0
B) equilibrated with 0.5M NaCl pH 4.0

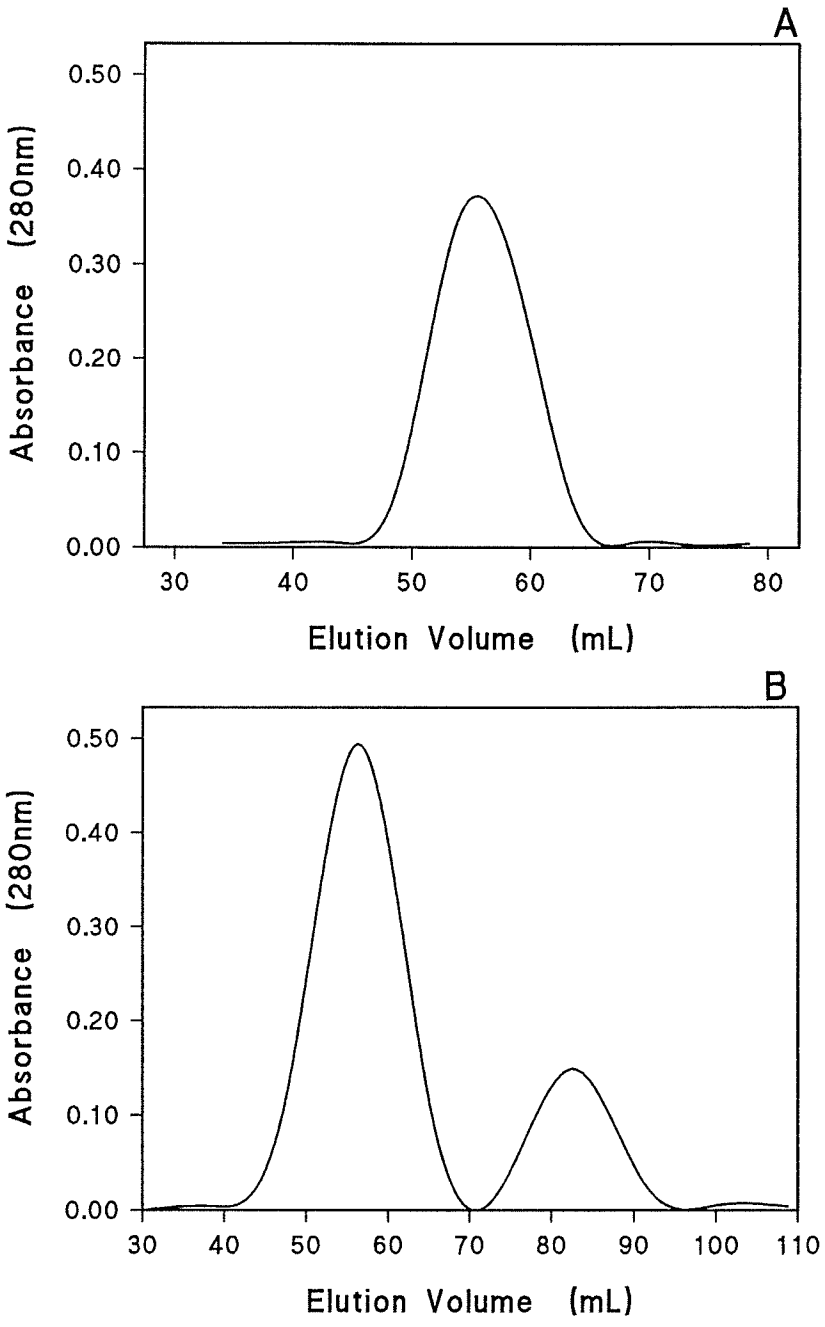

A
Absorbance (280nm)
0.50
0.40
0.30
0.20
0.10
0.00
30
40
50
60
70
80
Elution Volume (mL)
B
Absorbance (280nm)
0.50
0.40
0.30
0.20
0.10
0.00
30
40
50
60
70
80
90
100
110
Elution Volume (mL)

Table 24. The average molecular weights of the proteins eluted from the HiLoad 16/60 Superdex 200 Pharmacia column

Peak Number	Environments	
	0.5M NaCl pH 6.0	0.5M NaCl pH 4.0
1	274,000±18,400	221,000±18,800
2	-[1]	53,500± 1,890

[1] Second peak not present

purified protein to dissolve in the required solutions has made using this technique tediousand time-consuming to execute (Gill and Tung, 1976). As advised by Dr. LeJohn, gradient electrophoresis was used to determine the molecular weight of the main salt soluble canola protein.

b. Gradient Polyacrylamide Electrophoresis. As suggested by Dr. LeJohn (personal communication) gradient PAGE can be successfully used to determine molecular weight. When electrophoresing the peak (B) and shoulder (A) protein samples obtained from the S-300 chromatograph (Figure 3a), the molecular weight of the peak (Lane 3) was found to be 350,000±2,800, while the shoulder (Lane 2) contained two bands with molecular weights of 533,500±27,600 and 350,000±2,800 (Figure 7). In addition, the molecular weight of the proteins in various NaCl solutions (0.1M, 0.3M, and 0.5M) (Table 25) did not change in the various salt environments and only one band was noticed in all cases. The molecular weight in these salt environments was higher than the theoretical value proposed by Schwenke *et al.* (1983) (Table 2). The molecular weight of the diffuse experimental bands were determined at the midpoint of the band, as a result, some error was expected in the calculated molecular weight. When using this technique, a large measurable migration distances would separate proteins with molecular weights corresponding to 300,000 (12S globulin), 158,000 (7S or trimeric half molecule) and 50,000 (2-3S subunit). If present on the gradient gel (Figure 7), the 7S trimer would have been approximately 4cm from the 12S globulin. In latter runs the bovine serum albumin (64,000) standard was not allowed to run off the gel and no band was observed in the vicinity of serum albumin standard, so contamination with the 2-3S component was not expected. Based on these

Figure 7. The gradient polyacrylamide gel electrophoresis profile of the major salt soluble canola protein[1]

Lane 1. Pharmacia molecular weight standards
2. Shoulder portion (A) of the main peak[1]
3. Main peak (B)[1]

[1] Shoulder (A) and Main peak (B) fractions collected from the Sephadex S-300 gel filtration column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v) (Figure 3a)

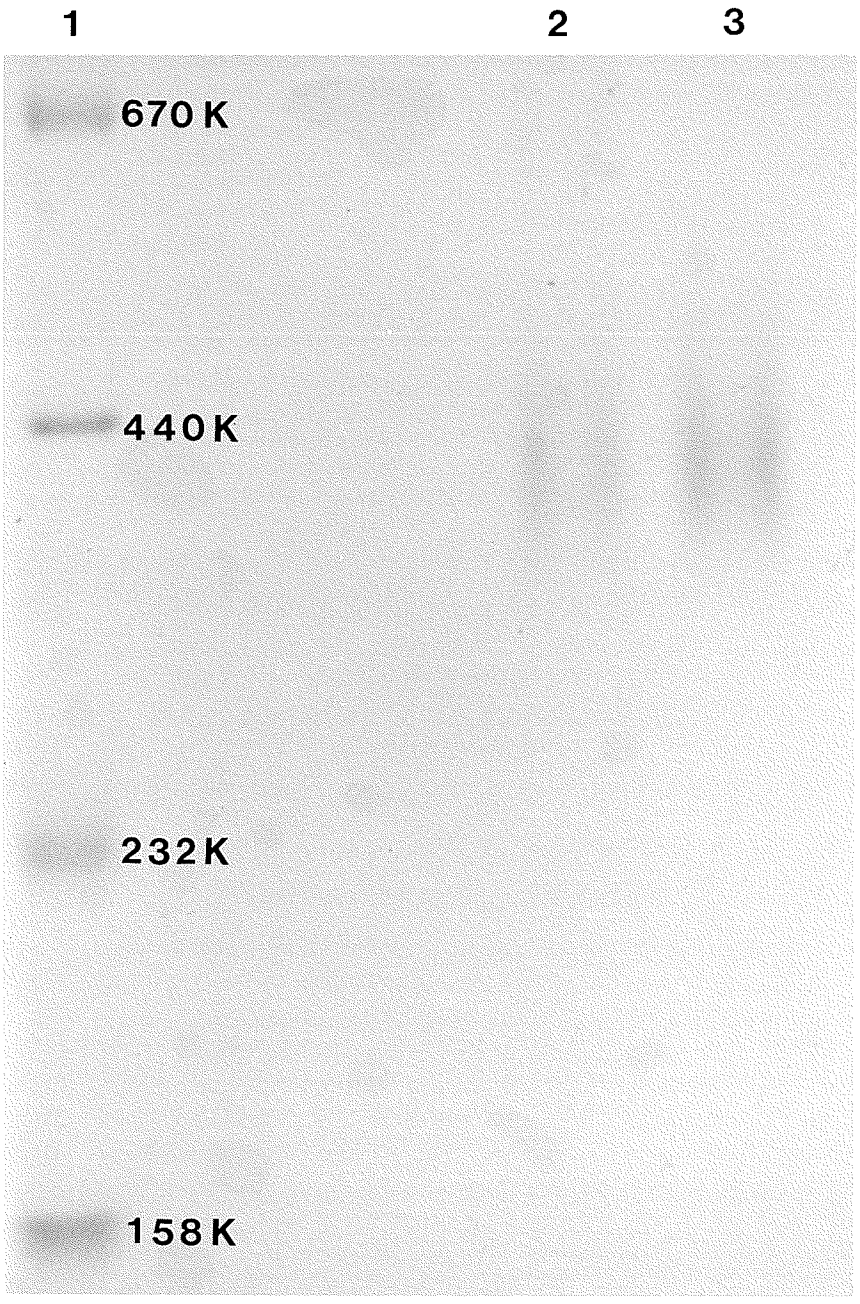

1
2
3
670K
440K
232K
158K

Table 25. Molecular weights of canola proteins estimated by gradient electrophoresis[1]

Molecular Weight			
Shoulder[1]	Main Peak[1]		
	0.1M NaCl	0.3M NaCl	0.5M NaCl
533,000±27,600	-[2]	-[2]	-[2]
350,000± 2,800	349,000±4,200	350,000±3,100	350,000±2,800

[1] Shoulder (A) and Main peak (B) fractions collected from the Sephadex S-300 gel filtration column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v) (Figure 3a)

[2] Protein band not observed

results, the protein appeared to be the pure 12S globulin, cruciferin.

2. Isoelectric Point Determination by Isoelectric Point Focusing

The isoelectric focusing profile of the main canola protein was determined at two different protein concentrations (0.5mg/mL and 1mg/mL) and four different molar concentrations of NaCl (0.05, 0.1, 0.3, and 0.5) in Figure 8 and Appendix F. Pharmacia (1982a) suggested salt levels exceeding 0.1M should be avoided as this can cause gradient distortion, but the type of salt also influences the degree of disturbances. Buffering salts (Tris and phosphates) cause considerably greater effects than NaCl (Pharmacia, 1982a). Despite these concerns, salt concentrations up to 0.5M NaCl did not induce wavy bands but the lower ionic strengths influenced the number of bands focused.

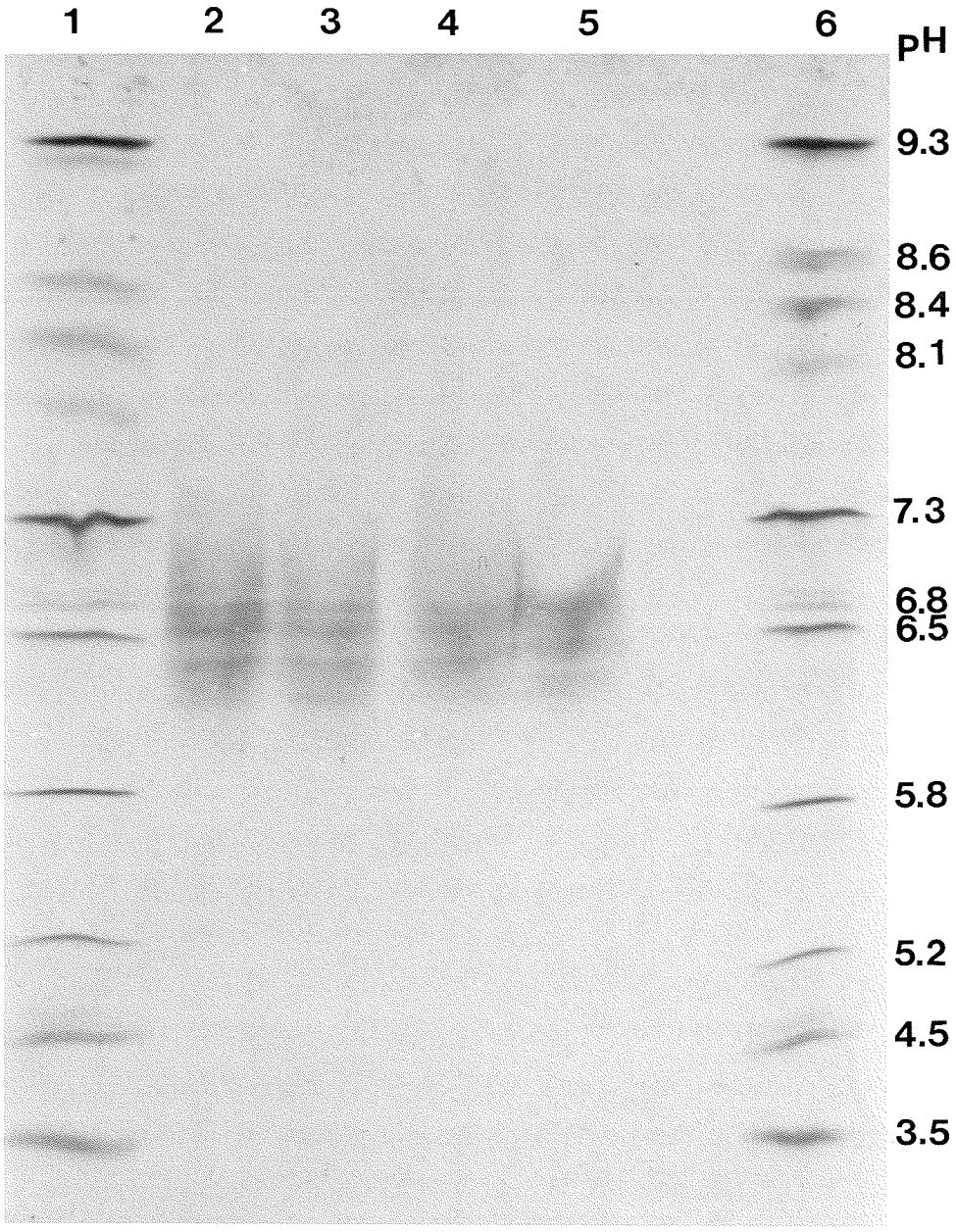
In all situations a number of bands were observed, as the ionic strength decreased from 0.5M to 0.05M NaCl more bands were observed. For the 0.5M protein sample the pI ranged from 6.6 to 7.2, this was increased to 6.2 and 7.42 for the 0.05M NaCl sample. Thus, the increase in range size appeared to be due to the decreasing ionic strength or possibly the protein was dissociating into its subunit components (7S or 2-3S) in the lower ionic strength environments. Similar multiple band profiles were observed in both the protein concentrations applied, the multiple banding patterns were not a function of protein concentration.

Many different explanations could explain the presence of these multiple bands. As the salt concentration deceased, the protein sample became hazy due to the presence of a precipitate. This precipitate in the

Figure 8. The isoelectric focusing profile of the major salt soluble canola protein dialysed in various NaCl environments[1]

Lane 1. Isoelectric Focusing standards (Table 7)
2. 0.05M NaCl protein sample
3. 0.1M NaCl protein sample
4. 0.3M NaCl protein sample
5. 0.5M NaCl protein sample
6. Isoelectric Focusing standards (Table 7)

[1] Main peak fraction (B) collected from the Sephadex S-300 gel filtration column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v) (Figure 3a). Portions of this collected sample were dialysed in 0.05M, 0.1M, 0.3M and 0.5M NaCl solutions for 24h.


1
2
3
4
5
6
pH
9.3
8.6
8.4
8.1
7.3
6.8
6.5
5.8
5.2
4.5
3.5

sample can cause trailing of the sample across the gel, resulting in a fuzzy pattern (Pharmacia, 1982a). This poor solubility was also indicated because all samples precipitated at the point of application. In initial runs, the protein was applied at approximately the pI, but sample precipitation at the site of application made it difficult to read the profiles. The protein was then applied closer to the anode, but the sample still precipitated and tailing also occurred. When the protein was applied in the pH range of 8-10, the protein may have been unstable and dissociated. The problem of globulins smearing was indicated in the literature. Gatehouse *et al.* (1980) indicated that pea protein (legumin) usually precipitated in the gel before it reached its pI, resulting in smearing instead of sharp bands. Since the molecular weight of the protein sample was high (approximately 300,000), the protein may have slowly penetrated into the gel and decreased the mobility of the sample in the gel. Multiple minor bands could result when the protein interacts with the ampholytes, small molecular weight polymers, which establish and maintain the pH gradient of the isoelectric focusing gel (Pharmacia, 1982a). These multiple peaks do not necessarily indicate sample denaturation or contamination.

Another reason for incorrect band positioning could be incomplete focusing. To remedy problem, a higher number of volthours could have been used. However, sparking along the gel edge was a problem, so increasing running time was not attempted. When Zarins *et al.* (1984) determined the pI of the 7S cottonseed globulin considerably longer running times were used at various currents (volts). After the gel was prefocused, the protein was applied and focused for 4h at 50V, 16h at 100V and finally to

sharpen the band 1h at 200V. The 7S globulin consisted of eight components in four doublets differing in charge with isoinic points ranging from pH 6.3 to 7.6 (Zarins *et al.* 1984). Thus even with long running times, multiple banding can occur.

In the literature little reference was paid to the pI of the 12S globulin and its dissociation components. An isoelectric point of the 7S or 2-3S globulins were not found in the literature. Using an LKB column in a sucrose density gradient (pH 3.5 to 10) at pH 4°C, Schwenke *et al.* (1981) determined the corrected pI to be 7.25±0.10 for the 12S globulin. When looking at a broad densitometer reading shoulders were evident on either side of the peak (Schwenke *et al.* 1981). Thus, some other pI values could also have been indicated.

Quinn and Jones (1976) performed urea isoelectric focusing using 6M urea on the 12S globulin extracted in water at various pH values. Despite problems in accurately assessing pI, due to interactions between urea and the protein as well as urea and carrier ampholytes, the majority of the pI values obtained were in the neutral range (6.0-7.3) which was comparable to the pH range found in this study.

3. Purity Assessment with Straight PAGE

In straight PAGE, when the main salt soluble canola protein was placed in an electric field, its electrophoretic mobility depended upon its charge and molecular weight. The support media or acrylamide gel has a specific pore size determined by the concentration of acrylamide in the gel. In the 5% gels the pore size is larger than in the 7.5% gels, thus, protein would migrate faster in the 5% gel because there is less sieving resistance as the protein migrated through the gel. In order to obtain

resistance as the protein migrated through the gel. In order to obtain better separation between high molecular weight proteins, the dye (bromophenol blue) was allowed to migrate off the gel during all the straight PAGE runs. Consequently, in this study the relative mobility (Rf) was not calculated for protein bands.

When the main peak (B) and its shoulder (B) illustrated in Figure 3a were electrophoresed two distinct bands were observed in the shoulder sample and only one broad band in the peak sample (Figure 9). The shoulder (A) contained a larger protein, likely aggregated 17S protein, which was not present in the main peak (B). Thus, the broad main peak was relatively pure. Of note, was the fact that in the main peak sample no band was observed across from the ovalbumin standard with a molecular weight of 44,000. Thus, the sample did not seem to be contaminated by the 2-3S fraction. Straight PAGE is generally run to assess purity and cannot be used to accurately determine the molecular weight of a protein. Since the thyroglobulin (670,000) and gammaglobulin (158,000) standards were poorly separated, it was not possible determine if the band corresponded to the 12S and or the 7S globulin protein based solely on the straight PAGE results.

In additional runs, the running time was increased and the gel concentration was increased to 7.5% with hopes of effectively separating the two higher molecular weight standards to give a better approximation of the molecular weight of the main salt soluble canola protein. When the samples from the 0.5M and 2.0M NaCl Sephacryl S-300 gel filtration columns were electrophoresed, very broad bands were observed in approximately the same location (Figure 10). These results suggested that under both

Figure 9. Straight polyacrylamide gel electrophoresis profile of the major salt soluble canola proteins[1]

Lane 1. Bio-Rad Molecular weight standards (Table 7)
2. Shoulder portion (A) of the main peak
3. Main peak (B)
4. Shoulder portion (A) of the main peak
5. Main peak (B)
6. Bio-Rad Molecular weight standards (Table 7)

[1] Shoulder (A) and Main peak (B) fractions collected from the Sephadex S-300 gel filtration column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v) (Figure 3a)

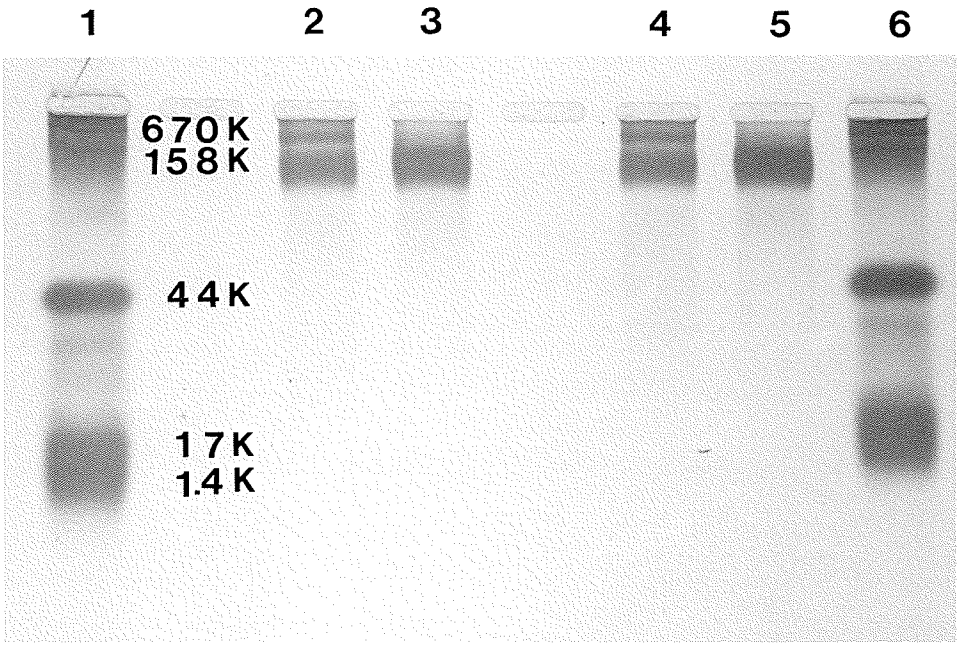
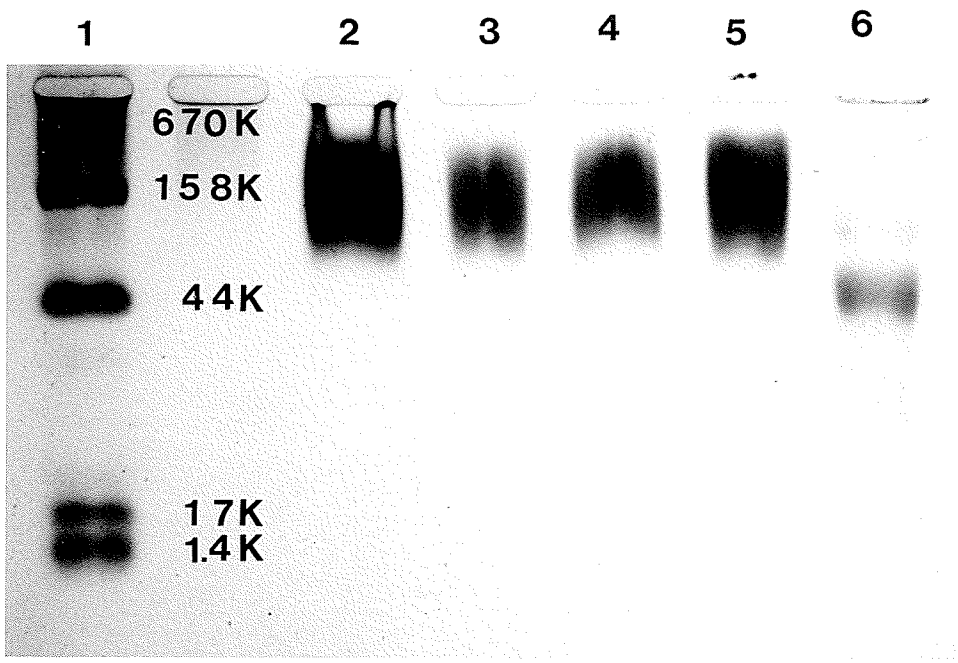

1
2
3
4
5
6
670K
158K
44K
17K
1.4K

Figure 10. Straight polyacrylamide gel electrophoresis profile of the major salt soluble canola proteins in 0.5M and 2.0M NaCl environments[1]

Lane 1. Bio-Rad gel filtration standards (Table 7)
2. 0.5M NaCl sample
3. 2.0M NaCl sample
4. 0.5M NaCl sample
5. 2.0M NaCl sample
6. Catalase (Pharmacia)

[1] Main peak fraction (B) collected from the Sephadex S-300 gel filtration column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v) (Figure 3a) and 2.0M NaCl with 0.2 sodium azide (w/v) (Figure 3b).


1
2
3
4
5
6
670K
158K
44K
17K
1.4K

estimate the molecular weight of the main salt soluble canola proteins extracted and purified in the 0.5M and 2.0M NaCl conditions as the two high molecular weight Bio-Rad standards (thyroglobulin and gammaglobulin) with the molecular weight difference of 522,000 were only separated by a few centimeters.

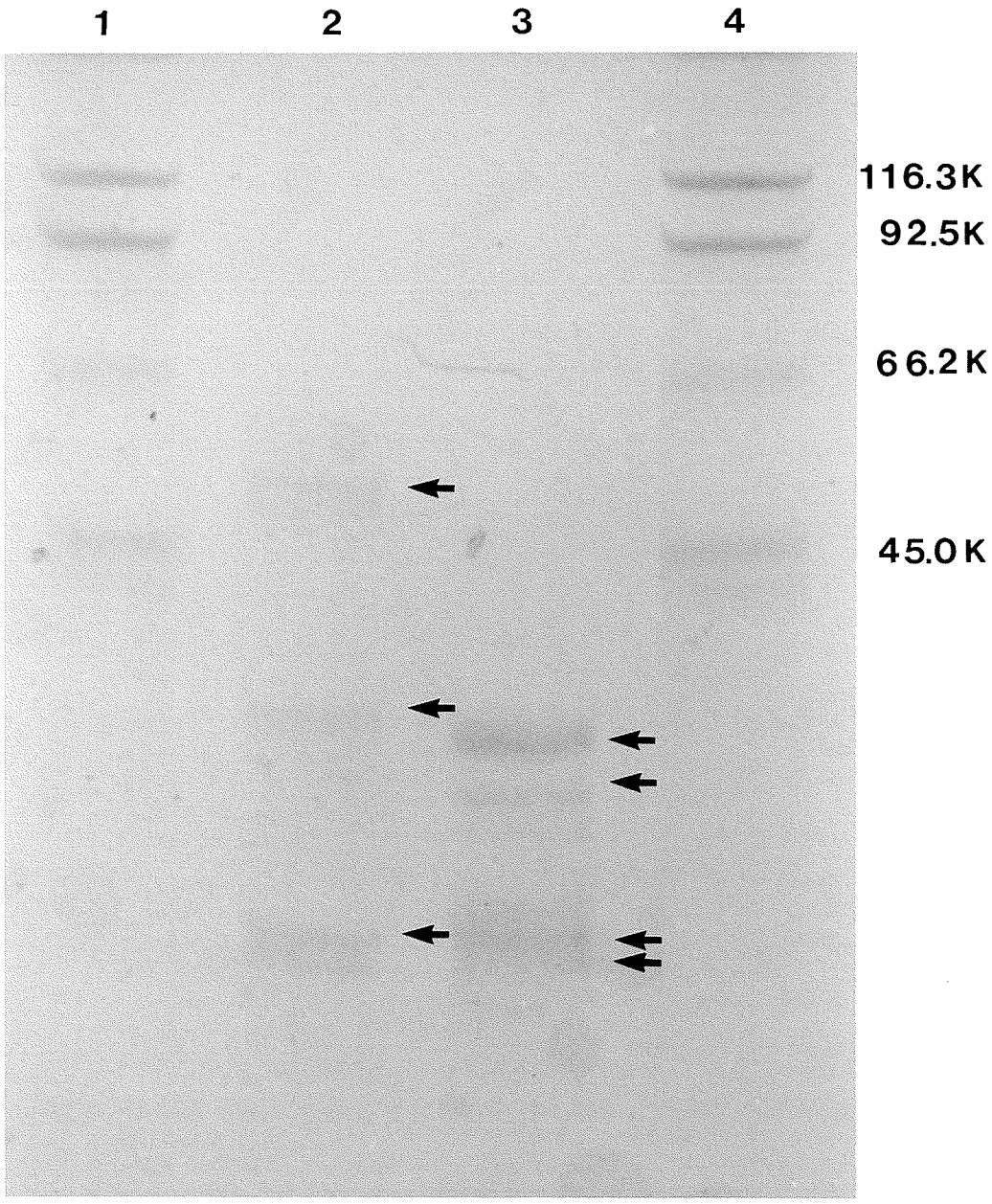
4. Subunit Analysis with SDS-PAGE. The canola fraction was analyzed by SDS-PAGE under reducing and nonreducing conditions. Sodium dodecyl sulphate (SDS) is a detergent which denatures the protein to its subunits by disrupting noncovalent links. The SDS binds to the protein to yield an uncoiled or rod-shaped protein with an excessive negative charge. The charge per subunit is constant, therefore the electrophoretic mobility of the protein-SDS complex is based solely on molecular weight of the subunit. In the protein sample containing mercaptoethanol, the covalent disulphide bonds between the acidic and basic subunits were also broken. The results are indicated in Appendix G and Figure 11. As anticipated in the sample without mercaptoethanol, a band with a molecular weight of 50,000 was found. This was anticipated and represented the 2-3S subunit. Unexpectingly however, four other bands were also observed. Somehow the covalent bond linking the acidic and basic polypeptides appeared to have been cleaved.

The SDS-PAGE analysis of the canola protein was performed under reducing conditions specifically to cleave the acidic and basic chains of the 2-3S subunit. Under these conditions, a set of bands at about 30,000 (acidic polypeptides) and another set at approximately 20,000 (basic polypeptides) were observed (Figure 11). These results were consistent

Figure 11. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the major salt soluble canola protein[1]

Lane 1. Pharmacia high molecular weight SDS-PAGE standards (β-galactosidase, phosphorylase β, bovine serum albumin and ovalbumin)
2. Sample without mercaptoethanol added
3. Sample with mercaptoethanol added

[1] Main peak fraction (B) collected from the Sephadex S-300 gel filtration column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v) (Figure 3a) and 2.0M NaCl with 0.2 sodium azide (w/v) (Figure 3b).


1
2
3
4
116.3K
92.5K
66.2K
45.0K

with the results of Gill and Tung (1978b) and Schwenke *et al.* (1983) (Table 26). The actual molecular weight of the acidic and basic subunits varied between these two studies, however, two sets of two distinct bands were observed, one with a larger molecular weight than the other. Despite, this discrepancy in the actual molecular weight of the subunits, the molecular weight of the subunits in this research seem consistent with the values in the literature.

5. Amino Acid Composition. The amino acid composition of the defatted canola meal, as well as the 12S globulin are shown in Table 27. Amino acid composition indicates that there are some differences in arginine, alanine, proline, tyrosine, valine and phenylalanine between the meal and the 12S globulin. The meal was rich in glutamic acid, alanine, proline, leucine and aspartic acid, while the 12S globulin was rich in glutamic acid, aspartic acid, valine, leucine and arginine. In correspondence to the research of Finlayson *et al.* (1968), Goding *et al.* (1970), Gill and Tung (1976) Schwenke *et al.* (1981), Prakash and Rao (1986), Norton (1989) and Ohlson (1985), the meal and 12S fraction contained high amounts of glutamic acid, aspartic acid and arginine and was low in sulfur containing amino acids. When looking specifically at the 12S globulin, the amino acid values are in reasonable agreement with those of Ohlson (1985) (Table 27). Overall the composition of five amino acids were high: glutamic acid, aspartic acid, arginine, valine and leucine, while cystine, methionine, tryptophan and histidine contents were low. In addition, the total essential amino acid content of the 12S

Table 26. Molecular weights of polypeptide chains of the 12S rapeseed globulin after denaturation in the presence of SDS and mercaptoethanol.

Source	PPC1	PPC2	PPC3	PPC4	2-3S
Gill and Tung (1978b)	12,200 ±1,000	17,400 ±400	30,300 ±1,100	37,300 ±400	-[3]
Schwenke *et al.* (1983)	18,500 ±800	21,000 ±500	26,800 ±900	31,200 ±1,600	50,000-53,000[4]
Canola Extract[1]	21,590 ±500	23,800 ±800	33,000 ±300	34,500 ±600	-[3]
Canola Extract[2]	33,200 ±1,500		23,000 ±900		50,000 ±300

[1] Molecular weights of the 12S globulin after denaturation in the presence of SDS and mercaptoethanol. (as calculated from Figure 11 Lane 3).

[2] Molecular weights of the 12S globulin in the presence of SDS in the absence of mercaptoethanol. (as calculated from Figure 11 Lane 2).

[3] The 2-3S globulin dissociates in the presence of mercaptoethanol.

[4] Based on the molecular weight determination of the 12S globulin in the presence of 8M urea (Simard *et al.* 1979 as cited by Schwenke *et al.* 1983).

TABLE 27. Amino acid composition of the Westar canola meal and the 12S canola globulin

AMINO ACID	MEAL (gAA/100g Protein)	12S (gAA/100g Protein)	12S[a] (gAA/100g Protein)
ASP	7.12	9.59	8.15
GLU	24.30	24.41	18.33
SER	5.02	4.59	5.09
GLY	5.79	4.43	5.50
HIS*	3.53	2.51	1.53
ARG*	4.93	7.27	7.33
THR*	4.47	3.94	4.79
ALA	8.01	3.72	4.99
PRO	7.46	3.72	6.21
TYR	1.78	3.08	3.26
VAL*	5.08	7.68	5.70
MET*	1.69	1.33	2.24
CYS	0.61	0.25	2.04
ILE*	3.74	4.77	4.48
LEU*	7.39	7.35	8.04
PHE*	2.97	5.28	3.87
LYS*	3.96	3.77	6.82
TRP*	2.14	2.32	1.63

* Essential amino acids
[a] Literature values (Ohlson, 1985)

globulin was 42.3mg/g which was larger than brown rice (40.5mg/g) and comparable to soybeans (43.0mg/g) and human milk (43.4mg/g) (Cheftel *et al.*, 1985)

Directly from the amino acid (AA) data, the average hydrophobicity (Hø), as well as the positive, negative and total charge potential, plus the frequency of charged groups were calculated (Table 28). The average hydrophobicity value (920 cal/AA residues) was lower than the value (1041 cal/AA residue) obtained by Schwenke *et al.* (1981) but comparable to 962 or 967 cal/AA residues observed by Prakash and Rao (1986) and Ohlson (1985) respectively. A higher negative and total potential, but a lower positive potential and identical charge frequencies were observed in comparison to the research of Ohlson (1985). When comparing the Hø with other 12S plant globulins in Table 4 the Hø was relatively high. The calculated partial specific volume (0.73mL/g) was identical to that quoted by Schwenke *et al.* (1981), Prakash and Rao (1986) and Ohlson (1985). The values determined for the Kjeldahl protein conversion factors in Table 28 were also comparable. It was interesting to note the estimated Kjeldahl protein conversion factor (5.92) for the meal was larger than the value (5.67) suggested by Mossé and Pernollet (1983). Using the 5.93 rather then 5.67 value would significantly increase the estimated protein in the meal from 35.6% to 37.2%. In addition, the estimated protein efficiency ratio (PER) value of 2.30 was the same as the soybean PER value (Cheftel *et al.* 1985) and comparable to the PER for casein (2.5) (Blaicher *et al.*, 1983). The estimated PER of the meal was 3.75, thus was higher than values for beef (3), cows milk (3.1), fish (3.5) and comparable to hen eggs (3.9) (Cheftel *et al.*, 1985). This unique PER, for a plant protein and a well rounded amino acid profile makes canola suitable for human consumption.

TABLE 28. Estimated parameters determined from the amino acid analysis of canola meal and the 12S globulin.

Protein Sample	$H\phi_{ave}$[1]	Potential[2]			Charge Frequency	PSV[3]	KPCF[4]	PER[5]
		(-)	(+)	Total				
Meal	826	224.74	80.27	246.20	0.302	0.73	5.92	3.75
12S[a]	920	243.66	85.67	270.51	0.348	0.73	6.03	2.30
12S[b]	967	182.47	96.82	220.47	0.349	0.73	5.82	2.68

1 Average hydrophobicity, (cal/AA residue).
2 Moles/100kg protein.
3 Partial specific volume, (mL/g).
4 Kjeldahl-protein conversion factor.
5 Protein efficiency ratio.
a Protein isolate in this study.
b Amino acid composition of the 12S globulin determined by Ohlson (1985).

As indicated by straight PAGE and gradient electrophoresis, the isolated protein characterised from both the 0.5M and 2.0M NaCl extracts were identical and apparently homogeneous. The protein consisted of two acidic (MW 33,000 or 34,500) and two basic (MW 21,500 or 23,800) subunits joined through a disulfide bond to form 2-3S monomers which noncovalently associated to form the polymeric protein. Using gradient electrophoresis, the protein was characterised by having an approximate molecular weight of 350,000. Despite the multiple banding observed during IEF, the estimated pI range of 6.6 to 7.2 was comparable to the 12S canola globulin. The protein was relatively hydrophobic as well contained high amounts of glutamic acid, aspartic acid and arginine and an estimated partial specific volume identical to value suggested by Schwenke *et al.* (1981). The properties of the 12S protein used in this study were comparable the those in the literature.

E. The Influence of Environmental Manipulation on Protein Conformation and Micelle Response

In the past, research has centered around the independent effect of pH and neutral salts on the micelle response of fababean proteins (Ismond *et al.*, 1986a; 1986b; Ismond *et al.*, 1990). Ismond *et al.* (1986a), manipulated the micelle response of vicilin in various environments and classified the response according to a visual rating scale. A broad spectrum of responses, ranging from discrete individual micelles to extensive networks or protein sheets, were observed where surrounding environment dramatically influenced the dynamic nature of the micelle response. The most appropriate environments for micelle formation and

eventual interaction were low concentrations (0.2 and 0.5M) of the moderately stabilizing anions ($C_2H_3O_2^-$, Br^-, Cl^-). Since moderately stabilizing anions were selected to induce optimal micelle associations in fababean proteins, NaCl was selected as the environment to study. Ionic strength and pH along with the protein concentration and dilution were modified to observe their impact on micelle response.

1. Types of Micelle Responses with the 12S Canola Globulin. In examining the micelle potential of the 12S globulin in a number of different conditions, it was apparent the micelle response was very dynamic. Due to the variable nature of the micelle response a basic definition was required:

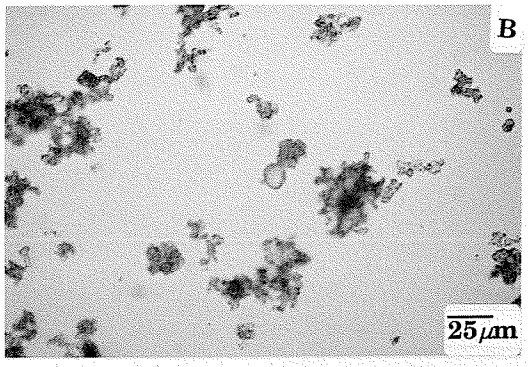
A. Micelle: is an aggregated product that forms when amphiphilic molecules are dissolved in an aqueous environment. These products are formed because a driving force, water, propels the hydrophobic residues of the protein to associate and form a hydrophobic core while hydrophilic residues are orientated towards the exterior of the protein surface (Tanford, 1973). (Rating 1; Figure 12A).

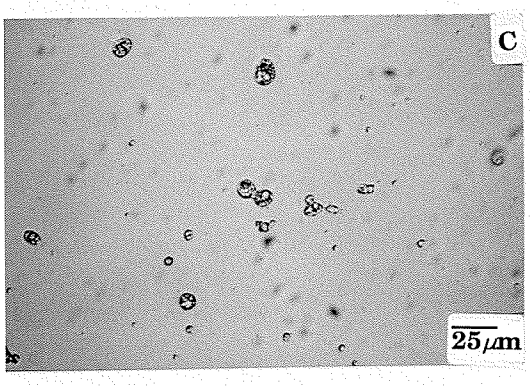
The amphiphilic nature of proteins makes them ideal for forming micelles, this is especially true of proteins with large amounts of hydrophobic residues. To reduce the overall energy of the system when added to aqueous solutions, the protein folds into a spherical configuration or micelle to bury most of the hydrophobic residues. Even though proteins structures are dynamic, their flexibility is limited by the polypeptide backbone which imposes steric constraints. As a result, it is physically impossible to bury all the hydrophobic residues in the interior, hence, hydrophobic patches remain on the protein surface. Consequently, protein micelles are more complex than the detergent

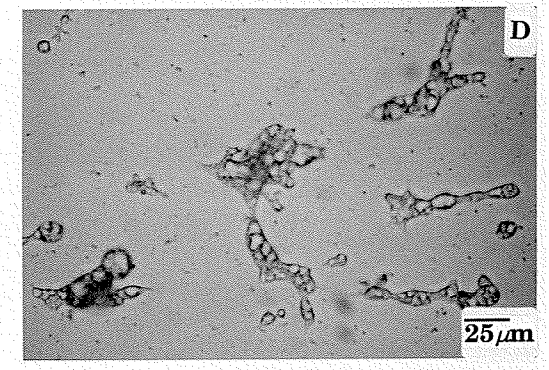
Figure 12. Photomicrographs of the micelle rating scale

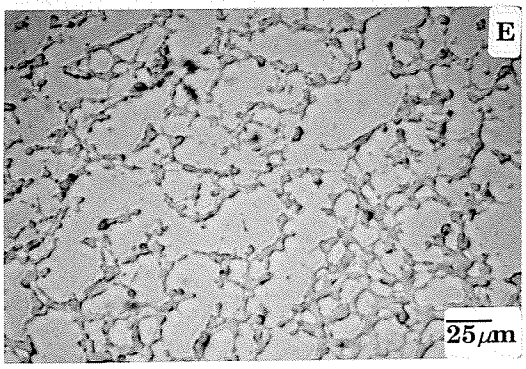
A.	Rating 1.	Small micelles.	(0.1M NaCl pH 7, 25mg/mL, 1:5)
B.	Rating 2.	Aggregation.	(0.5M NaCl pH 7, 25mg/mL, 1:15)
C.	Rating 3.	Coalescence.	(0.1M NaCl pH 7, 25mg/mL, 1:10)
D.	Rating 4.	Chaining.	(0.3M NaCl pH 7, 50mg/mL, 1:10)
E.	Rating 5a.	Networking.	(0.5M NaCl pH 6, 25mg/mL, 1:15)
F.	Rating 5b.	Sheeting.	(0.5M NaCl pH 6, 50mg/mL, 1:30)

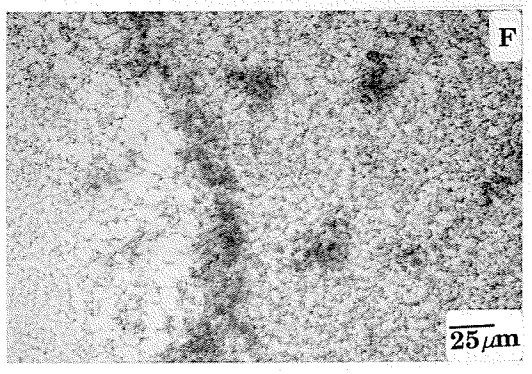

A
50 μm


B
25 μm


C
25 μm


D
25 μm


E
25 μm


F
25 μm

micelles defined by Tanford (1973).

B. Degree of Intermicellar Associations: When enough hydrophobic residues appear on the protein surface, hydrophobic patches are created (Fisher, 1965). These patches can be protected from the polar medium by self-association, thus, various degrees of intermicellar association are possible. Due to the diversity of the micelle response in various environmental conditions used in this study, some fundamental definitions were constructed.

1. **Aggregation:** is the uniting of protein into dense masses or bodies. The constituent protein clumps together but clumps remain relatively independent. This reaction may or may not involve the initial formation of micelles. (Rating 2; Figure 12B).

2. **Coalescence:** is the fusing or merging of two or more micelles to form a larger micelle. (Rating 3; Figure 12C).

3. **Chaining:** is an association that occurs when two or more micelles join together to form a straight or branched structures. (Rating 4; Figure 12D).

4. **Network:** is an interaction or joining of micelles into chains which are randomly or inextricably interwoven.(Rating 5; Figure 12E).

5. **Protein Sheets:** is an optimum micelle response which occurs when micelles interact to form a dense continuous thin layer. (Rating 5; Figure 12F).

6. **Micelle Response:** is a general concept referring to micelle formation or any degree of interaction.

Based on this descriptive classification (Table 29), photomicrographs (Figure 12) were selected to provide a visual representation of the micelle response. According to Tanford (1973), attractive hydrophobic force must predominate over repulsive forces in order for micelle formation (Rating 1) to occur. However, some surface

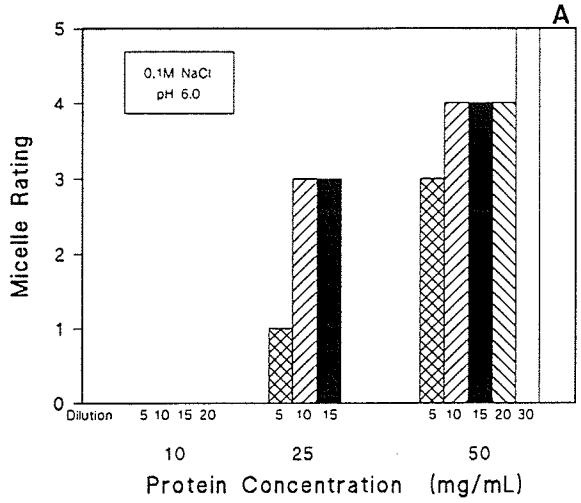
Table 29. A description of the visual rating scale

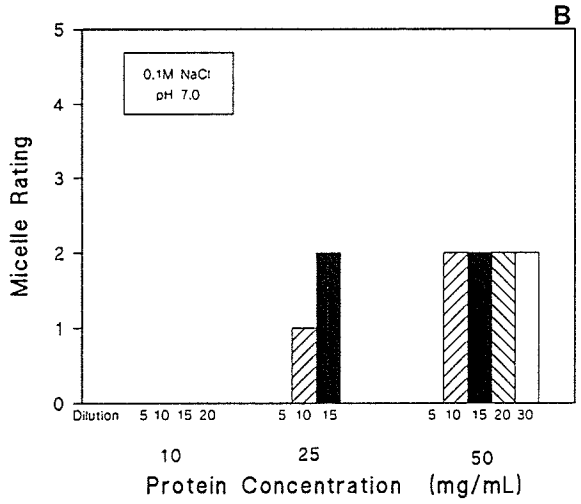
Characteristics	Micelle Rating
-no micelle formation or hazy background with no detectable micelles	0
-small discrete micelles	1
-aggregates of small micelles	2
-coalescence	3
-chaining	4
-networks and protein sheets	5

repulsion must be present to restrict micelle enlargement (Rating 3) and to enhance micelle interaction (Rating 4 & 5). In optimum environments, individual micelles were capable of extensive interactions resulting in the formation of elaborate protein networks and sheets. In many environments, aggregation was observed along with coalescence, chaining, networking or sheeting. As a result, the highest and most representative rating was assigned as indicated in Figures 13, 14, and 15. The surrounding environmental conditions were critical in determining the degree of interaction. The dynamic or broad response of the 12S canola globulin was dependent upon the original protein concentration, surrounding environment and dilution factor (Figures 13, 14, and 15). Studying these varying degrees of interaction will eventually provide insight for researchers on how to manipulate the micelle phenomenon to suit their needs.

To develop this insight, the noncovalent forces promoting these elaborate interactions are of fundamental concern and can be examined and manipulated with environmental changes. In this study, the impact of the environment on the micelle response was considered from two perspectives. Initially, the influence of specific environments (Table 19) on the conformation of the 12S globulin (Appendix K) or its dissociation products were examined. Conditions promoting extensive denaturation were expected to limit micelle formation and subsequent interaction. The molecular structure of the protein was assessed using DSC analysis (Figure 16a and 16b) and gel filtration, when molecular dissociation was suspected at pH 4.0 (Figure 6b). In addition, with light microscopy, the micelle response was examined in relation to pH, ionic strength, protein concentration and dilution factor (Figure 13, 14, and 15).

Figure 13. Effect of protein concentration and dilution on the micelle response in 0.1M NaCl at various pH values
A. pH 6.0
B. pH 7.0
C. pH 8.0


A
0.1M NaCl
pH 6.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15 20 5 10 15 5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)


B
0.1M NaCl
pH 7.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15 20 5 10 15 5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)

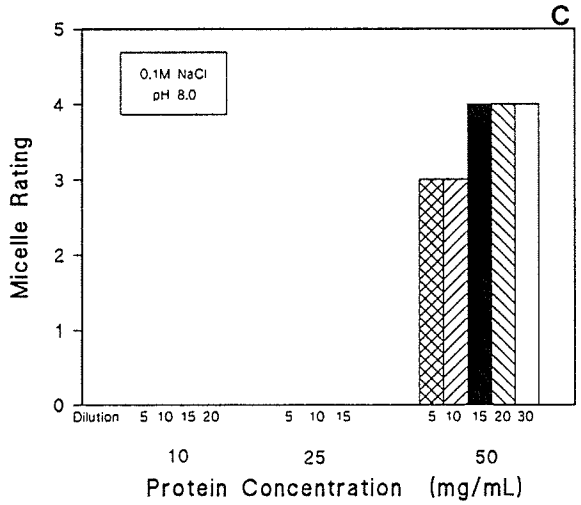
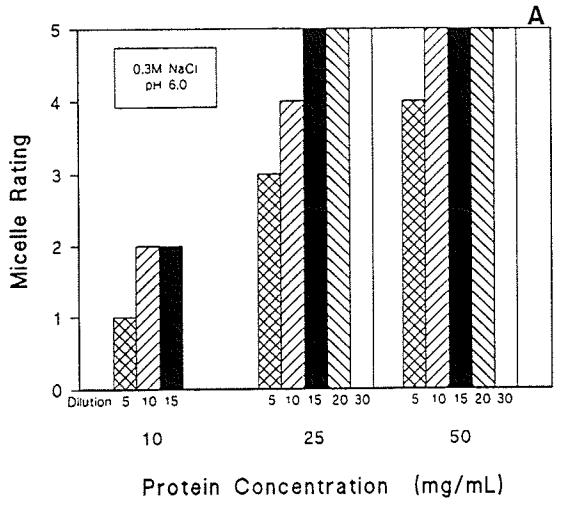
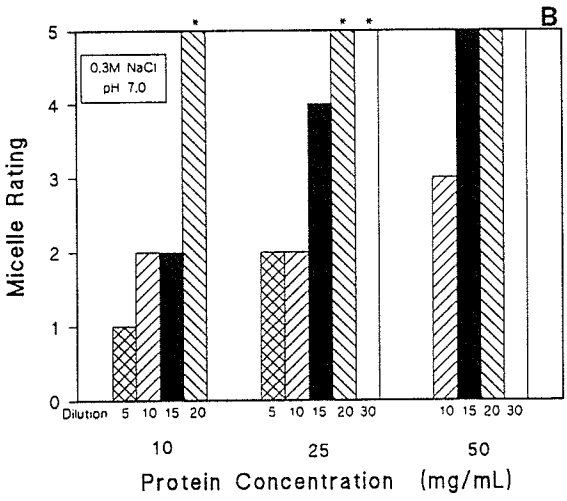

C
0.1M NaCl
pH 8.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15 20 5 10 15 5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)

Figure 14. Effect of protein concentration and dilution on the micelle response in 0.3M NaCl at various pH values
A. pH 6.0
B. pH 7.0
C. pH 8.0

*A very thin and fine or delicate protein sheet (Weak micelle rating of 5).


A
0.3M NaCl
pH 6.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15
5 10 15 20 30
5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)


B
0.3M NaCl
pH 7.0
Micelle Rating
0
1
2
3
4
5
*
* *
Dilution 5 10 15 20
5 10 15 20 30
10 15 20 30
10
25
50
Protein Concentration (mg/mL)

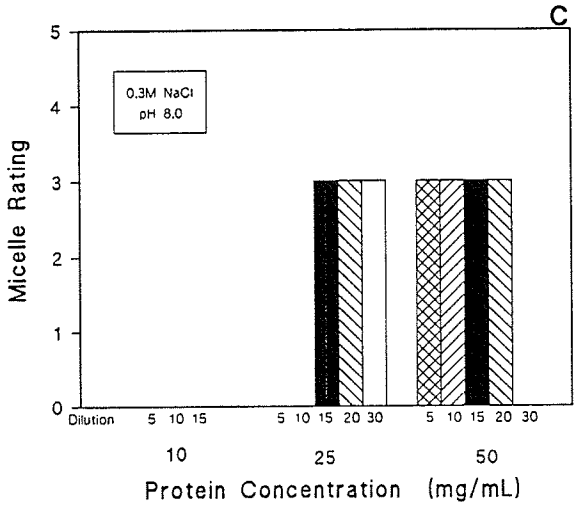
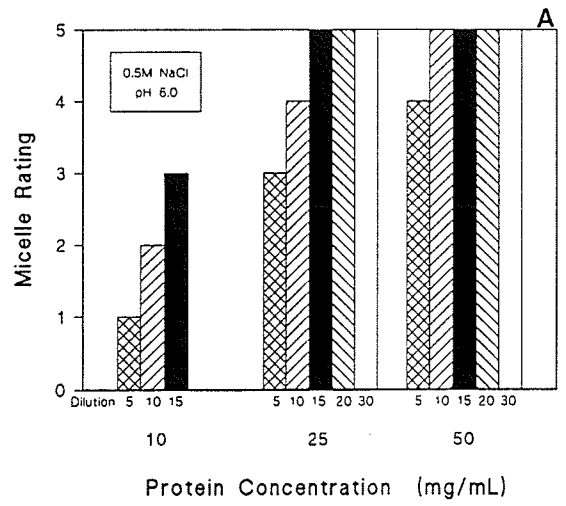
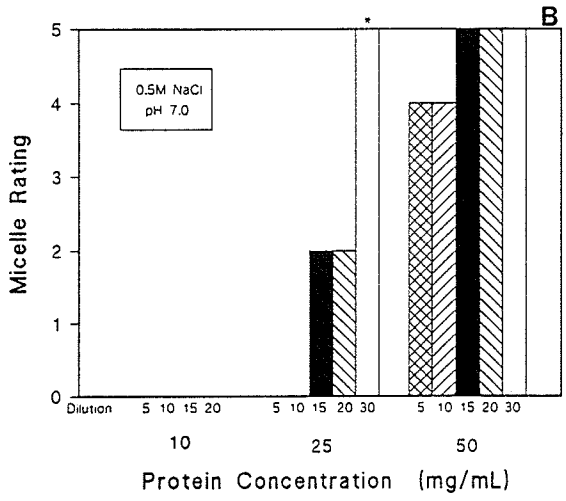

C
0.3M NaCl
pH 8.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15
5 10 15 20 30
5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)

Figure 15. Effect of protein concentration and dilution on the micelle response in 0.5M NaCl at various pH values
A. pH 6.0
B. pH 7.0
C. pH 8.0

*A very thin and fine or delicate protein sheet (Weak micelle rating of 5).


A
0.5M NaCl
pH 6.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15
5 10 15 20 30
5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)


B
*
0.5M NaCl
pH 7.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15 20
5 10 15 20 30
5 10 15 20 30
10
25
50
Protein Concentration (mg/mL)

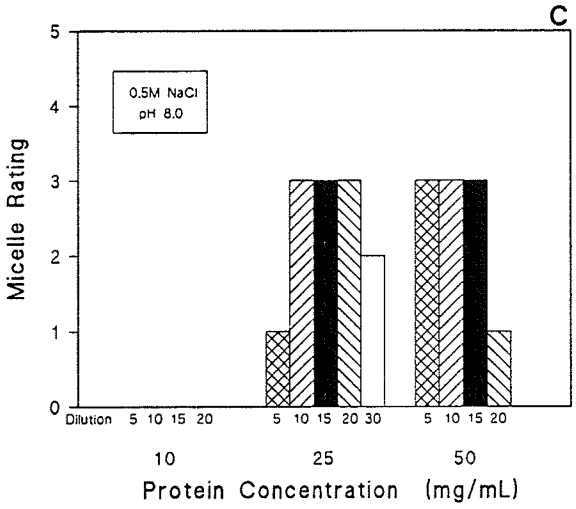
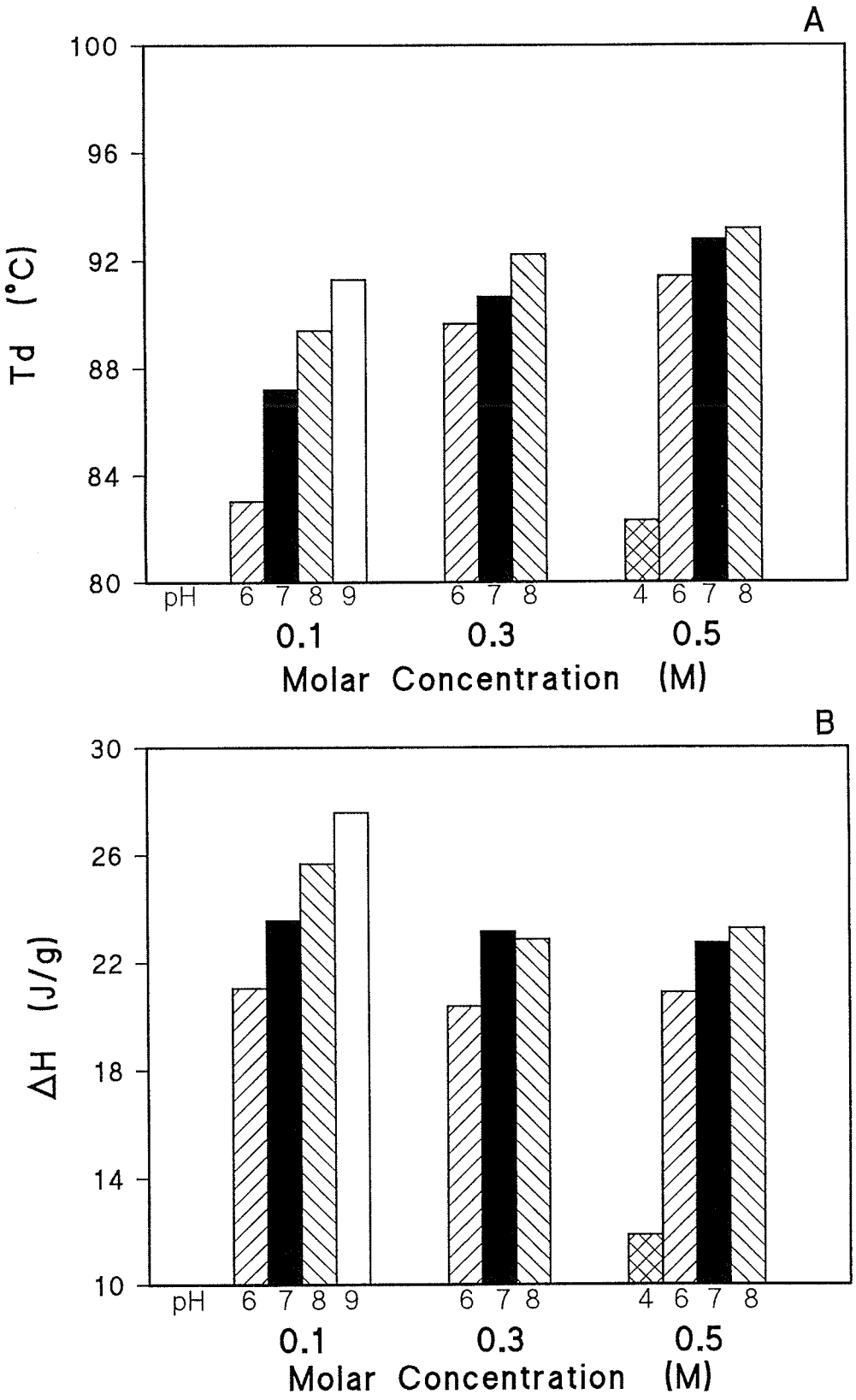

C
0.5M NaCl
pH 8.0
Micelle Rating
0
1
2
3
4
5
Dilution 5 10 15 20
5 10 15 20 30
5 10 15 20
10
25
50
Protein Concentration (mg/mL)

Figure 16. The effect of pH and salt concentration on the thermal properties of the major salt soluble proteins.
A). Thermal denaturation temperature (Td)
B). Enthalpy of denaturation (ΔH)


A
100
96
92
88
84
80
Td (°C)
pH 6 7 8 9
6 7 8
4 6 7 8
0.1
0.3
0.5
Molar Concentration (M)
B
30
26
22
18
14
10
ΔH (J/g)
pH 6 7 8 9
6 7 8
4 6 7 8
0.1
0.3
0.5
Molar Concentration (M)

2. Effect of Environmental Manipulation on Protein Conformation.

The overall spatial arrangement of globular proteins is generally very compact and globular, and within certain structural constraints the protein is able to conform with its surrounding environment. Thermal parameters (determined with DSC) are tools providing insight towards the degree of conformational change ranging from slight to drastic molecular changes in the presence of denaturating environments. One drawback of this technique is that a set protein concentration is required, hence, the effect of protein concentration or dilution cannot be assessed. In DSC, two parameters assess these structural changes. By indicating the temperature of denaturation, Td monitors molecular stability. The effects of pH and salt concentrations on the thermal properties of the major salt soluble proteins are illustrated in Figure 16a and 16b as well as Appendix K. Arntfield and Murray (1981) indicated this temperature was controlled by nonpolar hydrophobic interactions as well as the cooperativity between polar and nonpolar groups. The second thermal parameter, ΔH, measures the enthalpy required for thermal denaturation. Hence, any previous protein unfolding results in lower ΔH values. These thermal parameters provide an indication of the effect of the solvent on the protein conformation and a basis to monitor the influence of these changes on the micelle response.

In Figure 16a and 16b and Appendix L, the effects of pH and salt concentration on thermal properties (Td and ΔH) of the major salt soluble protein are illustrated. The Td values increased with increases in pH; however, no appreciable differences in ΔH values were observed except in 0.5M NaCl pH 4.0 where the ΔH value was significantly lower than at pH 6, 7, or 8 (Appendix Lb). Increasing the salt concentration from 0.1 to 0.5M

had no significant influence on the ΔH value while the Td values increased.

The insignificant variation in ΔH values (Appendix Lb) from pH 6-9 and from 0.1 to 0.5M NaCl, indicated that no major conformational alternations occurred over these ranges. However, at pH 4, the lower ΔH value indicated the protein conformation was no longer the same as at pH 6-9. In addition, gel filtration results from the Superdex column, equilibrated with 0.5M NaCl pH 4.0 (Figure 6b) demonstrated the protein had dissociated to its 2-3S form. At pH 4.0, repulsive electrostatic forces created by the net positive charge stressed the protein, caused it to unfold and dissociate. With increases in pH (6.0 to 9.0) and salt concentration (0.1M to 0.5M NaCl), drastic conformational changes did not occur. However, as implied by increasing Td values, increased molecular stability of the 12S globulin occurred as a result of slight and gradual distortions at the quaternary level. In the case of increasing NaCl concentration, the non-chaotropic effect (0.3M to 0.5M), of the moderately stabilizing chloride anion apparently caused the protein to fold and bury hydrophobic residues in response to an increase in surface tension in the aqueous protein environment.

The electrostatic influence created by changes in pH also seemed to stabilize the protein. As electrostatic repulsion tends to stress molecular conformation causing protein unfolding and solubilization, the increase in Td was not expected at pH 9.0. It would appear therefore that the 12S globulin was tolerant to alkaline pH levels up to and including pH 9.0.

3. Effect of Environmental Manipulation on Micelle Formation and Interaction.

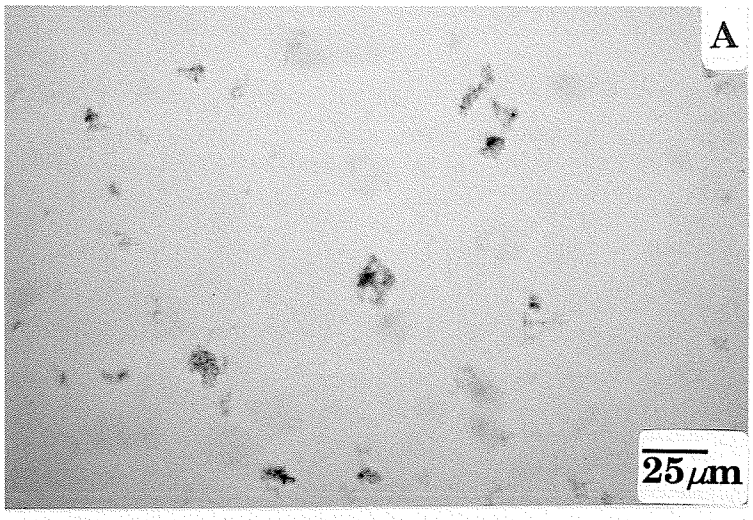
Since the surrounding environment can influence the ionization, noncovalent forces, conformation, and structure of the 12S canola globulin, it should also influence the micelle response. Thus, the micelle response depends upon the protein used and how it adapts to the surrounding environment. To better understand the complexity of the micelle response, each of the factors critical to the micelle response were considered in this study.

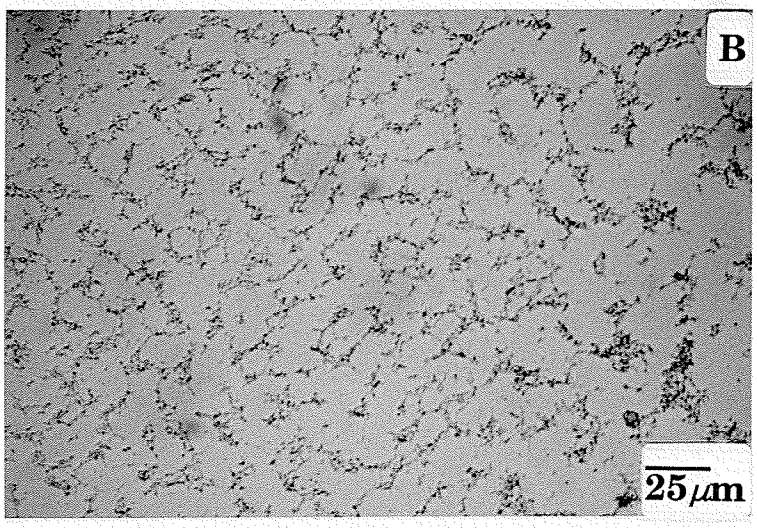
a. Protein Composition and Structure. The protein system used to elucidate the micelle response is important. In the current study, the high average hydrophobicity, the ratio of hydrophobic (aliphatic and aromatic) and polar residues, and flexibility of the 12S canola globulin were conducive to micelle formation and interaction. Generally, the native 12S canola globulin was able to form strong micelle responses. However, when the surrounding environment (for example 0.5M NaCl pH 4.0) caused the globulin to dissociate to the 2-3S form it was unable to elicit a micelle response.

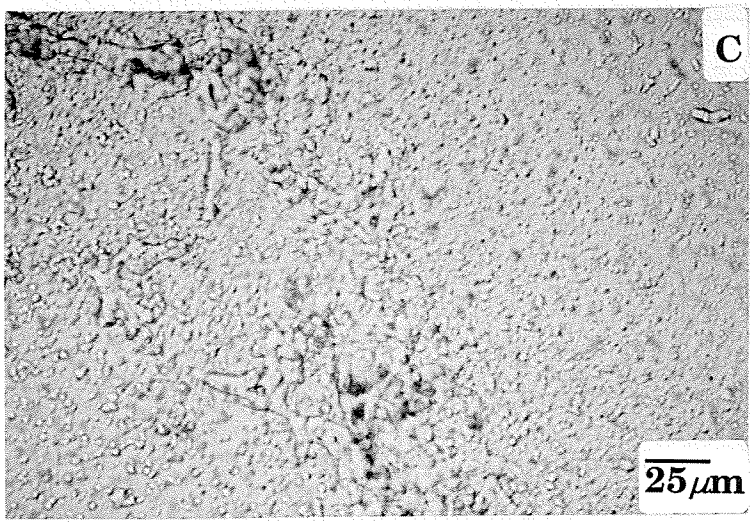
b. Critical Micelle Concentration (CMC). The CMC required to elucidate a micelle response depended upon the pH and ionic strength of the surrounding environment. In Figure 17, photomicrographs showing the effect of protein concentration in 0.5M NaCl pH 6.0 (1:15) are represented. As can be seen, micelle responses were observed over a broad concentration range; however, increasing the protein concentration improved the micelle response. Only the 0.3M NaCl (pH 6.0 and 7.0) and

Figure 17. Photomicrographs illustrating the effect of protein concentration on the micelle response in the presence of 0.5M NaCl pH 6.0 with a dilution factor of 1:15

A. 10mg/mL
B. 25mg/mL
C. 50mg/mL


A
25μm


B
25μm


C
25μm

0.5M NaCl (pH 6.0 and 7.0) environments were able to induce a micelle response in the 10mg/mL protein samples (Figure 13, 14, and 15). As the protein concentration increased to 25mg/mL, the observed micelle response in all environments increased except in 0.1M NaCl at pH 8 and 9 and 0.5M NaCl pH 4.0. At 50mg/mL, in 0.5M NaCl pH 4.0 or 0.1M pH 9.0 a micelle response was not visually apparent. While no absolute values for CMC were determined, it was generally observed that in low ionic strengths (0.1M) and extreme pH values (4.0, 8.0 and 9.0) the CMC was much lower than the 0.3 and 0.5M NaCl environments at pH 6.0 or 7.0.

c. Dilution Factor. The key step in the PMM procedure in isolating the solubilized hydrophobic plant proteins is the water dilution step. In aqueous milieu, hydrophobic interactions are a major forces promoting protein - protein interactions, responsible for micelle formation. The driving force for hydrophobic interactions arises not from the inherent attraction of the nonpolar surfaces but, with the energetically unfavorable effect they have on structure of the water molecules around them (Kauzmann, 1959). Since, it is physically impossible to internally bury all the hydrophobic residues, hydrophobic patches remain on the protein surface (Fisher, 1965). Therefore, the thermodynamically unfavorable interactions of the nonpolar patches with the water creates high systematic energy demands which are reduced through hydrophobic associations of non polar surfaces in close proximity (<3Å) (Murray *et al.*, 1981). As a result, the protein to water dilution factor tends to be the driving forces for micellization of amphiphilic molecules. The driving force must be sufficient to promote hydrophobic interactions,

however, excess water may dilute the protein concentration, thus, increasing the distance between protein molecules reducing the micelle forming potential.

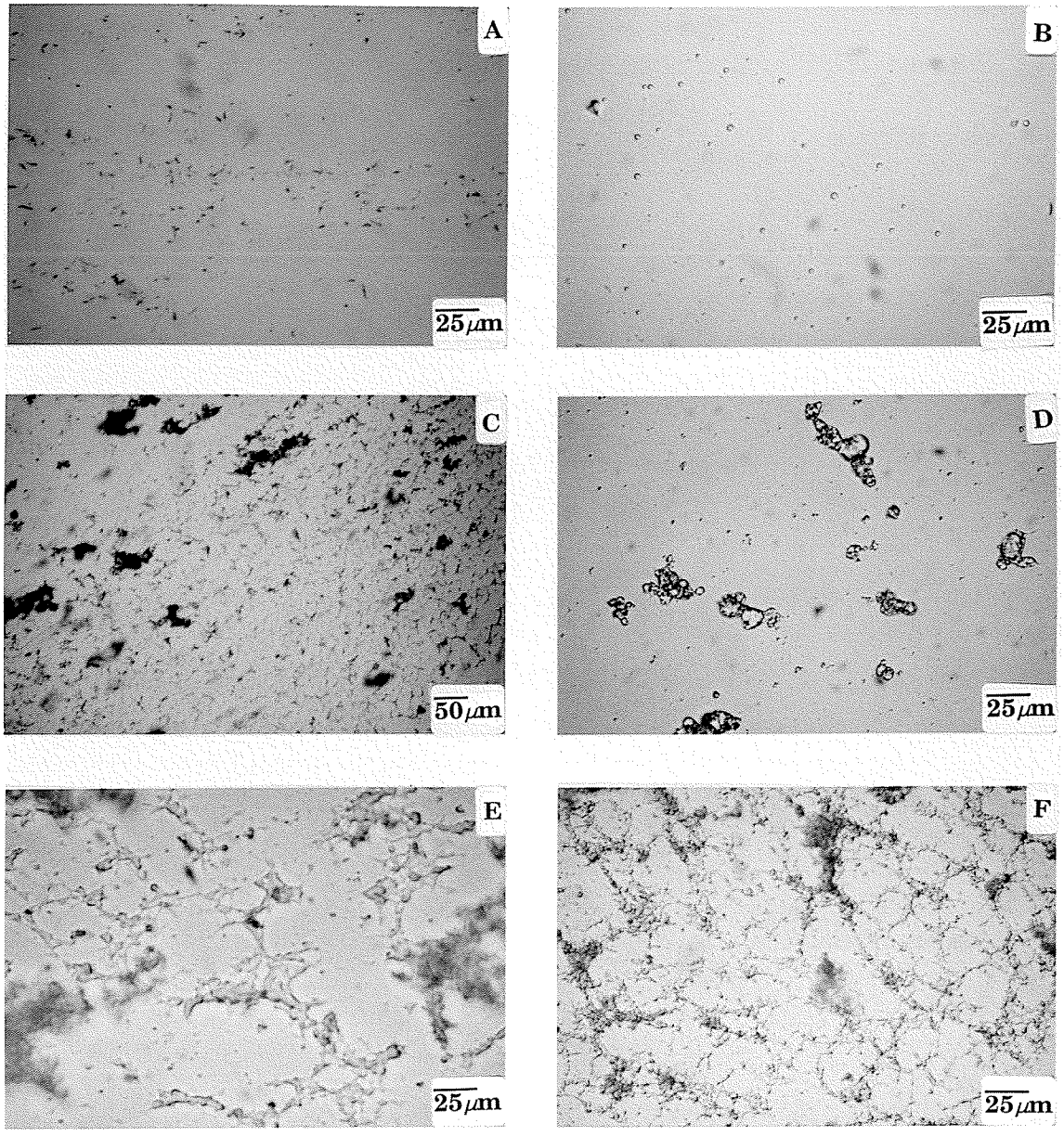
To show an example of the effect of dilution on micellization, 0.1M NaCl pH 6.0 (50mg/mL) was selected (Figure 18). Even though the sample contained precipitated protein prior to dilution (Figure 18a), micelle responses became stronger with increases in the dilution factor. For this system, dilutions were too low to demonstrate an inhibition of micelle formation due to reduced protein concentration.

In Figures 13, 14, and 15, the effect of the dilution factor (protein:water) on the micelle rating on 0.1M, 0.3M and 0.5M NaCl at pH 6,7, and 8 are shown. In viewing these Figures, generally, the optimum micelle responses were attained with dilutions as low as 1:15. In a few cases, the response improved slightly at higher dilutions (0.1M pH 6.0, 50mg/mL; pH 7.0 0.3M and 0.5M at 25mg/mL). In 0.3M NaCl pH 8.0 (50mg/mL), and 0.5M NaCl pH 8.0 (50mg/mL), the micelle response tended to decrease with an increase in the proportion of water (Figure 14c and 15c) showing the effect of reduced protein concentration. In most other environments, however, the micelle rating increased and then remained relatively unchanged with increasing dilutions.

Minimum dilutions for optimum response appeared to be a function of pH. At pH 6, the optimum response was obtained with a 1:10 dilution if the protein concentration was high enough. This optimum response was seen at a protein concentration of 50mg/mL with NaCl concentrations of 0.1M, 0.3M and 0.5M, as well as at 25mg/mL with 0.1M NaCl. At lower protein concentrations (10 and 25mg/mL), a dilution of 1:15 was required for

Figure 18. Photomicrographs illustrating the effect of dilution factor on the micelle response in the 0.1M NaCl pH 6.0 (50mg/mL) environment.

A. Predilution
B. 1:5
C. 1:10
D. 1:15
E. 1:20
F. 1:30


A
25 μm
B
25 μm
C
50 μm
D
25 μm
E
25 μm
F
25 μm

comparable responses.

A similar trend was seen at pH 7 (Figure 13b, 14b, and 15b), except higher dilutions were required to induce similar micelle ratings. At protein concentrations of 50mg/mL (0.3M and 0.5M NaCl), and 25mg/mL (0.1M NaCl), a 1:15 dilution was required while at concentrations of 10 and 25mg/mL (0.3M and 0.5M NaCl), a 1:20 dilution was necessary. The one exception to this trend was the 50mg/mL sample in 0.1M NaCl where the response was similar (rating 2) at 1:10, 1:15 and 1:20 dilutions but seemed to improve with a 1:30 dilution.

At pH 8, the maximum response was lower than at the other two pH levels (Figure 13c, 14c,and 15c). Furthermore, the dilution effect seemed to be dependent on the ionic strength of the system. With 0.1M NaCl, a concentration of 50mg/mL was required to give a response. In this environment, the effect of dilution was minimal because the micelle ratings increased from 3 with dilutions of 1:5 to 1:10 dilutions and to a 4 rating at higher dilutions. At higher ionic strengths, there was an initial increase in the micelle response with an increase in dilution factor, but this rating either levelled off without forming networks (0.3M NaCl, 25mg/mL) or decreased as the dilution factor increased (0.3M NaCl, 50mg/mL; 0.5M NaCl, 25mg/mL and 50mg/mL). In fact, the decrease was such that at 50mg/mL for both the 0.3M and 0.5M NaCl environments, no micelle response was obtained. Although, the maximum response was attained at relatively low dilutions (1:5-1:15) in the pH 8 environment, the micelle response was low (3-4). The inability to elicit a micelle response at the higher dilutions emphasized the instability of the system and the potential for protein solubilization if the dilution factor is too great.

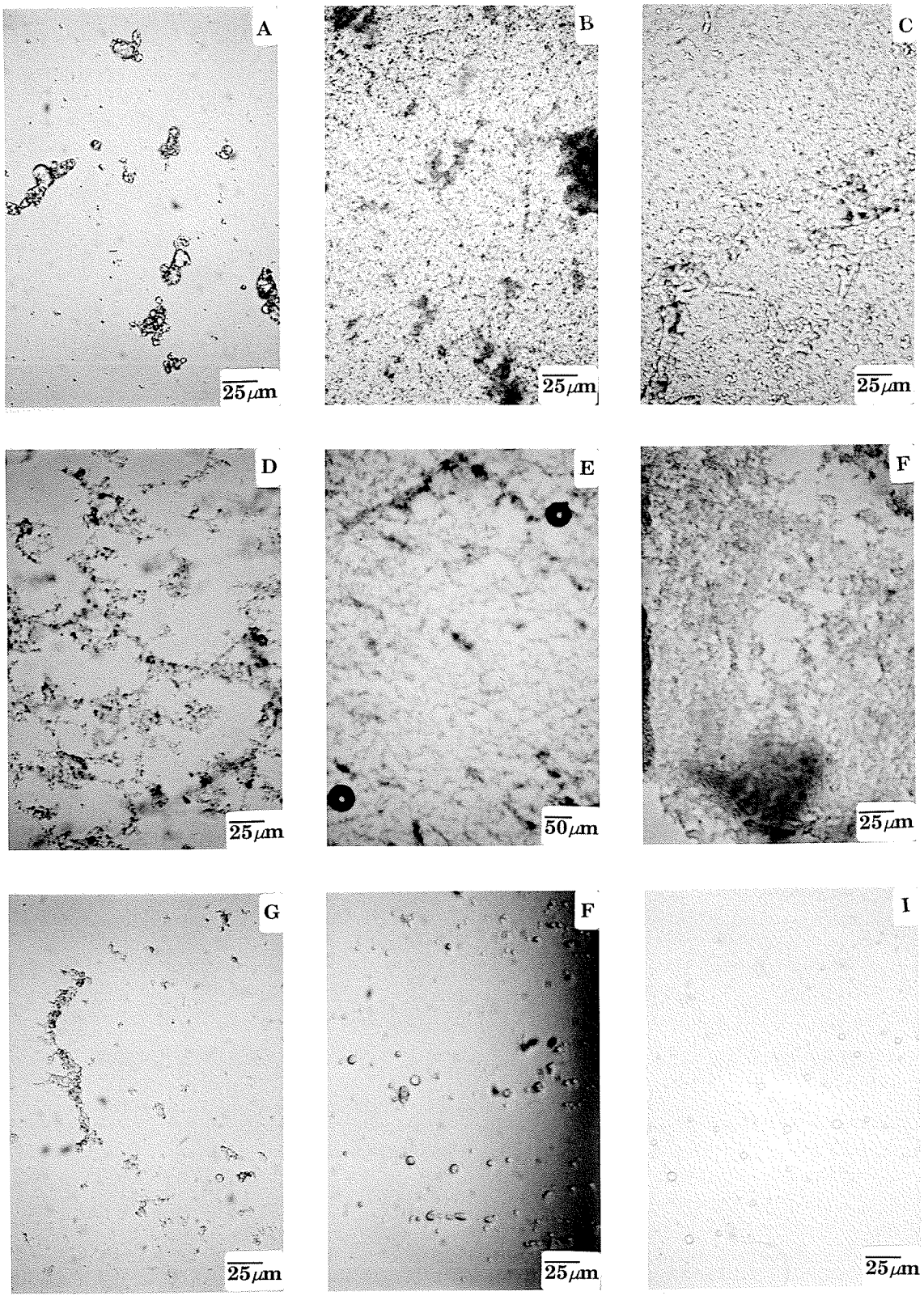
Clearly, the necessary dilution ratio is dependent on pH and protein concentration. The effect of protein concentration on the required dilution was noticeable in all environments in that the higher protein concentrations required lower dilution ratios to give comparable or better responses. The pH effect was especially evident by the different relationship between dilution and micelle response seen at pH 8 compared to 6 and 7. Even when comparing the responses at pH 6 and 7, it was apparent that for comparable protein concentrations and ionic strengths, the dilution ratio required to give optimal responses was always lower at pH 6.

d. Effect of pH. Proteins are polyelectrolytes since ionizable groups from the C and N terminal residues along with side chain residues participate in acid - base equilibrium (Cheftel *et al.*, 1985). As a result, protein titration curves are very complex because constituent amino acids are ionized over a wide pH range. The pK values of each amino acid can vary considerably depending on local environments. To make matters more complex, the proximity of other ionized groups, hydrophobic residues or H bonds also effect these curves. In aqueous environments, the majority of ionizable groups are located at the protein surface and hydrophobic residues buried internally. As a result, changes in pH can effect the overall protein charge and conformation which in turn should influence the micelle response.

The results in Figures 13, 14, 15 and 19 indicate pH played a critical role in determining the micelle response. The extreme pH environments (4, 9) were destructive to the micelle response. In 0.5M

Figure 19. Photomicrographs illustrating the effects of pH (vertically) and NaCl salt concentration (horizontally) with 50mg/mL samples diluted 15 times with water (Bars is equivalent to the represents the

A. 0.1M NaCl pH 6.0
B. 0.3M NaCl pH 6.0
C. 0.5M NaCl pH 6.0
D. 0.1M NaCl pH 7.0
E. 0.3M NaCl pH 7.0
F. 0.5M NaCl pH 7.0
G. 0.1M NaCl pH 8.0
H. 0.3M NaCl pH 8.0
I. 0.5M NaCl pH 8.0


A
25 μm
B
25 μm
C
25 μm
D
25 μm
E
50 μm
F
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25 μm

NaCl pH 4.0, the protein dissociated and was unable to elucidate a micelle response. While in 0.1M NaCl pH 9.0 a hazy background was observed, however, no discrete micelles were identified, even with phase contrast microscopy. In the pH 8.0 environments, the highest micelle rating (4) was observed in 0.3M NaCl (50mg/mL; 1:10) and 0.1M NaCl (50mg/mL; 1:15) while in 0.5M NaCl (50mg/mL) the highest observed rating was three. Even under optimum conditions of dilution, concentration and ionic strength, the micelle rating was less than desirable. Only in pH 6 and 7 environments (0.3M and 0.5M NaCl) were extensive networks and protein sheets observed. In addition, the pH 6.0 environment tended to elucidate strong micelle ratings over a broader range of experimental conditions than the pH 7.0 environments.

By investigating the effects of pH on the micelle response, some insight was gained into the impact of noncovalent forces on this response. Since the goal of the dilution step of the PMM procedure was to obtain maximum protein precipitation, conditions facilitating precipitation must be considered. Proteins, amphoteric molecules, tend to precipitate when electrically neutral and pH manipulation influences protein conformation which in turn has direct repercussions on the micelle phenomenon. In this study, the pI of the 12S globulin ranged from 6.57 to 7.19. Consequently, unlike the pH 7.0 environments, the protein would have a slight positive charge in the pH 6.0 solution. This appeared to be important in eliciting a stronger response over the examined experimental conditions. This was especially apparent when comparing 0.1M and 0.5M NaCl environments (Figure 13a and 15a). As a result, a slight electrostatic charge must be present to give the critical hydrophilic - hydrophobic balance required for a

strong micelle response. This is in agreement with the results seen for fababean proteins as reported by Ismond *et al.* (1986a; 1990).

The importance of this balance was further realized as the pH increased above or below the pI. As the pH increased to 8.0 and 9.0 or declined to pH 4.0, localized charges (net negative or positive) repulsed each other and stressed the molecular conformation resulting in unfolded or solubilized protein. At pH 4.0, the ΔH value from DSC and gel chromatographic results indicated the 12S globulin had unfolded and dissociated to its 2-3S monomeric form. This molecular destabilization had a detrimental effect on the micelle response and not capable of producing any micelle response at the conditions investigated. Under these conditions, the 2-3S monomer does not seeem as capable as the 12S globulin in the formation of micelles.

This high net negative charge at pH 8 and 9.0 was also incapable of inducing a strong micelle rating, despite the fact that no major conformational changes were noted with the thermal analysis. The increased Td values in these environments were indicative of protein stabilization possibly through a burial of hydrophobic residues. As reflected by ΔH, the protein did not unfold, however, slight conformational changes occurred as indicated by the Td values (Appendix L). Looking specifically at pH 8.0 environments, the optimal responses were characterised by ratings of 3 or 4 (Figure 13c, 14c, and 15c). Only under a few optimal conditions did micelles form chains. Generally, the increase in repulsive forces together with an increased burial of hydrophobic residues suppressed the hydrophobic interactions between micelles by not allowing the hydrophobic patches to come in close enough

proximity to allow micelle coalescence or chain formation. As the pH further increased to pH 9.0 (0.1M NaCl), no response was observed, even though thermal parameters again indicated no major structural alternations. Thus, the large electronegative force was detrimental to the response because it assisted in solubilizing the protein and suppressing the hydrophobic interactions, even though the overall protein structure was not altered. To elucidate a strong micelle response, therefore, pH extremes (4, 8, 9) should be avoided because net positive or negative charged protein systems tend to undesirably precipitate or solubilize the protein.

Based on this research, some general observations could be made in regards to the effect of pH. With canola globulins, the protein conformation limits are important, since unfolding or dissociating to monomeric subunits was detrimental to micelle formation. Therefore, conditions initiating such drastic conformational changes should be avoided. A second consideration in micelle association is that molecular surface properties and protein flexibility may play an important role. It has been suggested that proteins must be flexible to allow slight orientational changes of the hydrophobic and hydrophilic residues without disrupting the overall conformation of the protein. This oscillation of residues is important to generate a specific distribution of hydrophilic and hydrophobic residues where the hydrophobic attractive forces predominate over the electrostatic forces and result in the uniting of hydrophobic patches within close proximity (Murray *et al.*, 1981). In this research, the poor response at pH 8 and 9 may partially reflect the decreased flexibility. The thermal properties indicated a more stable

molecule at these elevated pH values without major conformational change. Thermodynamically, this would involve increased burial of hydrophobic residues and a less flexible molecule which would account for the poor micelle response. Finally, the overall charge of the protein is important. A slight repulsive force is necessary to initiate chaining and eventual sheet formation, this electrostatic force must exist or micelles would continue to coalesce to form larger micelles rather than form interactive networks. Increasing overall protein charge too high (positive or negative), however, limited the micelle response by dissociating or solubilizing the protein. In this research, it was difficult to determine the relative importance of a negative or positive charge on the micelle response. Ideally, to do this pH values equidistant from the pI should be investigated. As the pI of the 12S canola globulin ranged from 6.57 to 7.19, the net positive charge at pH 6 would probably not be as great as the net negative charge at pH 8. This difference however would not be large. Therefore, it would appear that coalescence, seen at pH 8 instead of networking and sheeting, may be partially due to the presence of the net negative charge rather than the size of the net charge.

e. Ionic Strength. To understand the effect of NaCl concentration on the micelle response, the relationship between ionic strength and protein structure must be considered. Generally, there are two ways in which salts can impact proteins. At low ionic strengths, there is generally a solubilizing or salting-in effect due to a change in the electrostatic interactions. At higher ionic strengths, the prominate influence is

through a modification of solvent environment rather than a direct effect on protein charge. In discussing the impact of ionic strength on the formation and interaction of protein micelles, these two different mechanisms must be considered.

Data for micelle formation at various NaCl concentrations is shown in Figures 13, 14, 15 and 19. At low salt concentrations (0.1M NaCl), there was noticeable protein precipitation prior to dilution (Figure 18). It is possible that ionic strength was not sufficiently high to optimize the salting-in. This pre-dilution aggregation, however, could help explain the poor micelle response of the 0.1M environmental conditions (Figure 13a, 13b, and 13c). Nevertheless, there was evidence of micelle formation and interaction if the protein concentration was high enough. Due to the electrostatic effect at this salt level, the protein should be less compact and possibly slightly unfolded in comparison to proteins subjected to higher salt concentrations. Thus, at low salt concentrations, more hydrophobic sites would be orientated towards the exterior of the protein surface. Upon dilution, the electrical double layer around the protein would disrupt (Ismond *et al.*, 1986b) in response to the polar aqueous environment and the protein would interact to bury its hydrophobic sites, thus, decreasing the entropy of the system. In other words hydrophobic associations between molecules are thermodynamically favored during dilution. However, the low initial solubility along with the high electrostatic forces which predominate at low salt concentrations hindered further micelle interaction.

Since electrostatic influences depend upon the polyionic nature of the protein which changes with pH fluctuations, the pH of the surrounding

environment was also important. At pH 7.0, the protein was relatively neutral and was further inclined to precipitate; hence, even less protein was available for hydrophobic interaction upon dilution. Consequently, the potential for micelle formation was hindered even more. Even high dilutions and protein concentrations were not capable of producing a rating greater than 2. At pH 8 and 9, canola proteins possess a net negative charge and are highly soluble (Prakash and Rao, 1986). At pH 9.0, the effect of these electrostatic forces was so devastating even high protein concentrations and dilution ratios were not sufficient to induce the hydrophobic interactions necessary for micelle formation. Upon dilution the background became hazy, however, no discrete micelles were observed. In the presence of high protein concentrations (50mg/mL), the micelle ratings of 4 were observed at pH 8. However, the repulsive forces seemed to decrease the potential for elaborate networking.

At pH 6.0, the 12S globulin possesses an overall net positive charge increasing the potential for binding the Cl^-. With increasing protein concentrations, the relative binding effect of the Cl^- would be smaller as the Cl^- to protein ratio would decrease. This slight increase in positive charge at increased protein concentrations and high dilutions, resulted in stronger micelle responses (Figure 13a). Consequently, the electrostatic effects on the micelle surface which hinder micelle formation and interaction were minimized by the binding potential of the Cl^-. In comparison to the other 0.1M NaCl environments in Figure 13b and 13c, the pH 6.0 milieu induced stronger micelle responses at lower protein concentrations and dilutions. For example, when comparing the 50mg/mL samples at pH 6 and 8, a micelle response of 4 was observed at a 1:10

dilution at pH 6, while the micelle response was 3 in pH 8.0. At the highest dilution, in the pH 6 environment, elaborate networks were formed while only intricate chaining was observed in pH 8.

With increases in NaCl concentration to 0.3M and 0.5M NaCl, the impact of NaCl on proteins through solvent modification was also a factor. The molecular stabilization resulting from these levels of NaCl was demonstrated by increased Td values (Appendix H). This capacity of the Cl^- to exert a stabilizing influence on the protein surface is related to its ability to increase the surface tension of the protein aqueous environment (Melander and Horvath, 1977) and cause preferential hydration of the protein molecule (Arakawa and Timasheff, 1982). In addition, the Cl^- has the ability to bind to proteins, especially at high concentration (Scatchard *et al.* 1957 as cited by Ismond *et al.* 1986b). This binding of the Cl^- may limit preferential hydration, which forces hydrophobic residues to orientate towards the interior of the protein. Therefore, there would still be nonpolar residues available for self association. When comparing 0.3M NaCl and 0.5M NaCl, in an environment where the protein had a net negative charge (pH 8), an interesting micelle response trend was noted. Generally, in these environments (except for 0.5M pH 8.0 25mg/mL) the initial the micelle rating of 3 remained unchanged and then decreased (Figure 14c and 15c). As the ionic strength increased to 0.5M, deterioration in the micelle response at the dilution extremes was even greater. The trend was further accentuated at low protein concentrations (25mg/mL). Clearly, the repulsive forces, and the orientation and concentration of the hydrophobic residues were not conducive to strong micelle responses.

At pH 7.0, where the charge on the protein was relatively neutral, the ionic strength played a dramatic role in determining the observed micelle response. The 0.3M NaCl environment was much more effective in producing a micelle response at lower protein concentrations than in 0.5M NaCl (Figure 14b and 15b). With protein concentrations of 25mg/mL, both environments were capable of forming very thin, fine and delicate networks and sheets. However, with the 0.5M NaCl environment no response was observed until dilution of 1:15 where ratings were lower for comparable dilutions. At a higher protein concentration (50mg/mL), strong networks and sheets were observed for both salt concentrations with dilutions as low as 1:15. The superiority of the 0.3M NaCl environment at pH 7.0 may be related to a pre-dilution precipitation problem with 0.5M NaCl. Upon dilution extensive protein aggregation was observed along with the micelle response.

At pH 6.0, the effect of ionic strength on the micelle response was not as evident as at pH 7.0. Here, the micelle responses were virtually the same over the observed experimental conditions (Figure 14a and 15a). Consistently, these two environments produced the strongest micelle responses. High initial solubility, slight repulsive charges, and a hydrophobic balance, appeared to induce strong micelle responses. When the hydrophilic - hydrophobic balance was optimal, as observed at pH 6 in high salt conditions, micelle responses could be observed even at low protein concentrations and dilutions.

After studying the influence of environmental manipulation on protein conformation and micelle capacity, it was evident that in addition

to a "native" 12S globulin, a delicate hydrophilic - hydrophobic balance was critical to micelle formation and subsequent interaction. Generally, with these experimental environments, the response, at any given protein concentration, depended upon the pH and ionic strength of the solubilizing environment as well as the dilution factor. The dilution factor was the thermodynamic driving force for hydrophobic association of the nonpolar patches in close proximity. In most environments, the micelle rating increased and then remained relatively unchanged with increasing water dilutions. An exception to this was at pH 8.0 (0.3M NaCl, 50mg/mL; 0.5M NaCl 25mg/mL and 50mg/mL), where the maximum response was attained at relatively low dilutions (1:5 - 1:15) and then declined with higher dilutions. Consequently, the solubilizing medium had a dramatic effect on the potential of the system to form micelles and produce elaborate networks or protein sheets. In low ionic strengths (0.1M NaCl), the micelle formation capacity was limited because the protein precipitated prior to dilution. Despite this, the ΔH values remained relatively high (roughly 24J/g) and were similar to ΔH values at higher NaCl concentrations. Near the pI, (pH 7.0), only protein aggregation was observed in this low ionic strength environment; even high dilutions could not induce micelle formation. Even though micelle responses improved at pH 6.0 and 8.0, the solubility problems of the 0.1M NaCl environments inhibited the broad responses observed at the higher ionic strengths (0.3M and 0.5M NaCl). As a result, low ionic strength should be avoided when optimizing protein isolation with the PMM procedure.

With increases in salt concentration to 0.3M or 0.5M NaCl, the effect of ionic strength was not as critical as the pH. Due to the

repulsive effect and high solubility of canola globulins at pH 8.0, initial micelle ratings (3) deteriorated with dilutions above 1:20 (0.3M NaCl 50mg/mL; 0.5M NaCl 25mg/mL and 50mg/mL). The ionic strength had a greater impact at pH 7.0, where the micelle response was better than at pH 8.0, but less than pH 6.0. Visually, the micelle response at pH 7.0 was different from pH 8 or 6.0 as the micelles remained small and numerous aggregations were observed along with any of the micelle response. Here, the chains and sheets looked like precipitated or aggregated protein joined together resulting in higher micelle ratings. Visually, these responses were not as desirable as those at pH 6.0. The protein sheets observed at 25mg/mL (Figure 14b and 15b) were so fine and delicate that they were barely visible with the light microscope. Although, the ratings at pH 7.0 were comparable to those at pH 6.0 the quality of the networks was not as high. With 0.5M NaCl, some protein precipitation was observed prior to dilution due to the combination of low solubility at the pI and the salting out effect created by the high Cl^- concentrations. However, if protein concentration and dilution factor was high enough, the exposed hydrophobic residues were thermodynamically driven to interact and form chains or protein sheets. Even though the 0.3M NaCl pH 7.0 environments produced stronger responses than the 0.5M environment, the strength of these responses were less than observed at pH 6.0 in that a higher dilution was required to get a similar response. Thus, in order to optimize the PMM procedure and minimize the dilution required, the protein should be solubilized at pH 6.0 in high ionic strength media. Either 0.3M or 0.5M NaCl would do as the micelle responses were virtually the same. (Figure 14a and 15a).

Clearly, the conditions solubilizing the protein dramatically influenced the micelle response for any given protein concentration and dilution factor. Under optimal conditions, lower protein concentrations and dilution factors were required to induce stronger micelle responses. The protein sheets contained few aggregates and were so dense that photomicrographs had to be taken at the edge of the sheet. When considering the commercial application of the PMM procedure, the pH 6.0 environment at high ionic strengths should be utilized. These conditions are advantageous for a number of reasons. A lower protein concentration means less energy expenditure, and smaller dilution factors infer less water required, as well as less centrifugation time and energy are required to recover the PMM.

CONCLUSIONS AND RECOMMENDATIONS

Increasing the efficiency, feasibility and consequently the marketability of the PMM procedure for isolating canola proteins was the goal of this research. By scaling up the methodology of Welsh (1988), through higher centrifugation speeds and better protein concentrating techniques, the overall recoveries increased from approximately 4% to 31%. Further research focused on the adaptation of the PMM process to the physicochemical properties of canola proteins to further optimize protein recovery. Two key steps in this methodology were scrutinized, the extractability of the protein and the dilution of the high salt protein extract (HSPE).

To maximize the extractability of the protein from the meal, two solubility studies were conducted on the meal. After the first solubility studies, a conclusion was drawn that the initial extraction step could be improved by increasing the NaCl concentration from 0.1M to 0.5M representing an increase in protein solubility of roughly 20%. Further increases in NaCl did not significantly increase protein solubility; however, optimal conditions were attained with both the 0.5M and 2.0M NaCl extractions conditions.

Replacement of NaCl with SHMP resulted in no significant increase in protein solubility. In view of the need to subsequently remove the phosphate ion and the reportedly low pH necessary to precipitate protein from this environment (Thompson *et al.* 1976), no further consideration was

given to the use of SHMP in the PMM procedure. Upon characterization, it was shown that both the 0.5M and 2.0M conditions selectively isolated the same protein. Since the relative extractabilities with these NaCl conditions were the same and purification in 2.0M NaCl was difficult, the 0.5M NaCl extraction was preferred for further work. The isolated protein was identified as the 12S globulin with a molecular weight of 350,000 (gradient PAGE). It represented approximately 60% of the material applied to the gel filtration column. Presumably, the remaining material was albumins.

The micelle phenomenon of the 12S globulin was complex, dynamic and was determined by the initial protein concentration, pH and ionic strength of the solubilizing environment as well as the dilutions factor. Generally, the micelle response at any protein concentration depended on the surrounding environmental conditions; however, the micelle response in all environments increased with increases in protein concentration. The surrounding environment influenced the surface properties of the protein and therefore, the micelle response. As in the fababean research, a hydrophobic - hydrophilic balance including slight repulsive electrostatic forces were important for micelle formation and interaction. The surface properties of the 12S globulin required to initiate micelle formation and interaction were optimal in 0.3M and 0.5M NaCl at pH 6.0. In extreme pH environments (pH 4, 8, or 9), the strong repulsive forces inhibited the micelle response while at the pI, pH 7.0, the repulsive forces were negligible and enhanced precipitation (0.1M and 0.5M NaCl) prior to dilution. In low ionic strength environments (0.1M NaCl), solubility problems hindered micellization. Upon dilution the main attractive force

promoting the micelle response appeared to be hydrophobic, therefore, the protein to water ratio was very important and depended on the surrounding milieu. Thus, this driving force and the exposure of a certain number of exposed hydrophobic residues appeared to be critical for intermicellar association. In view of data presented, it is plausible to suggest the next step of the PMM isolation procedure should be to perform and evaluate the procedure under environmental conditions of 0.3M and 0.5M NaCl at pH 6.0 while comparing the effects of dilution factors of 10, 15 or 20. The color, sensory evaluation, feeding trials, levels of antinutritional factors, and protein content of the isolate could all be evaluated. To accurately estimate and compare the protein recoveries between replicate or environments, the protein concentration prior to dilution should be monitored and controlled. This would help to compare protein recoveries between replicates or different environmental conditions. The quality, stability and functionality of the isolate should also be assessed. In so doing, this research would further determine the feasibility of the PMM procedure for commercial application.

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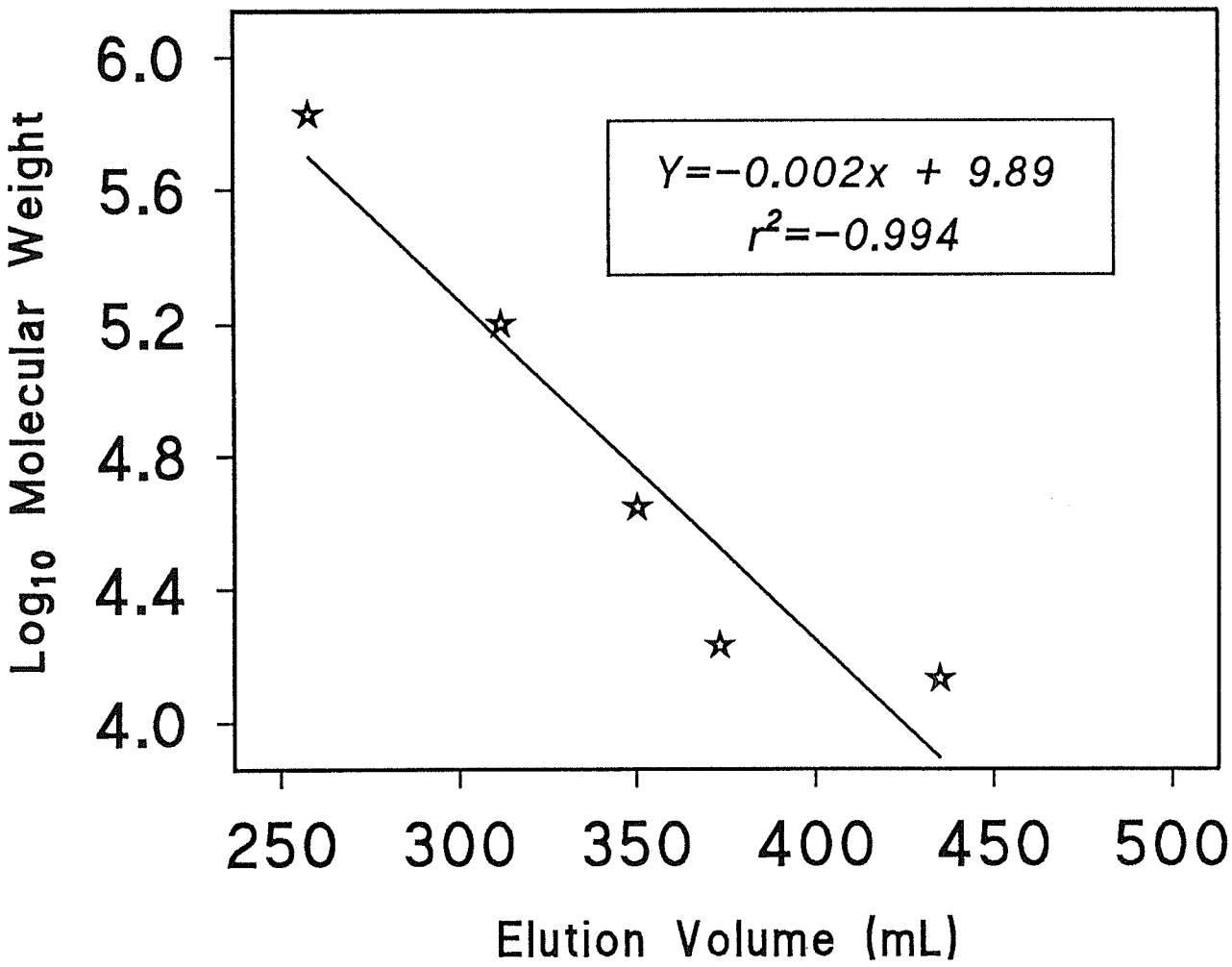
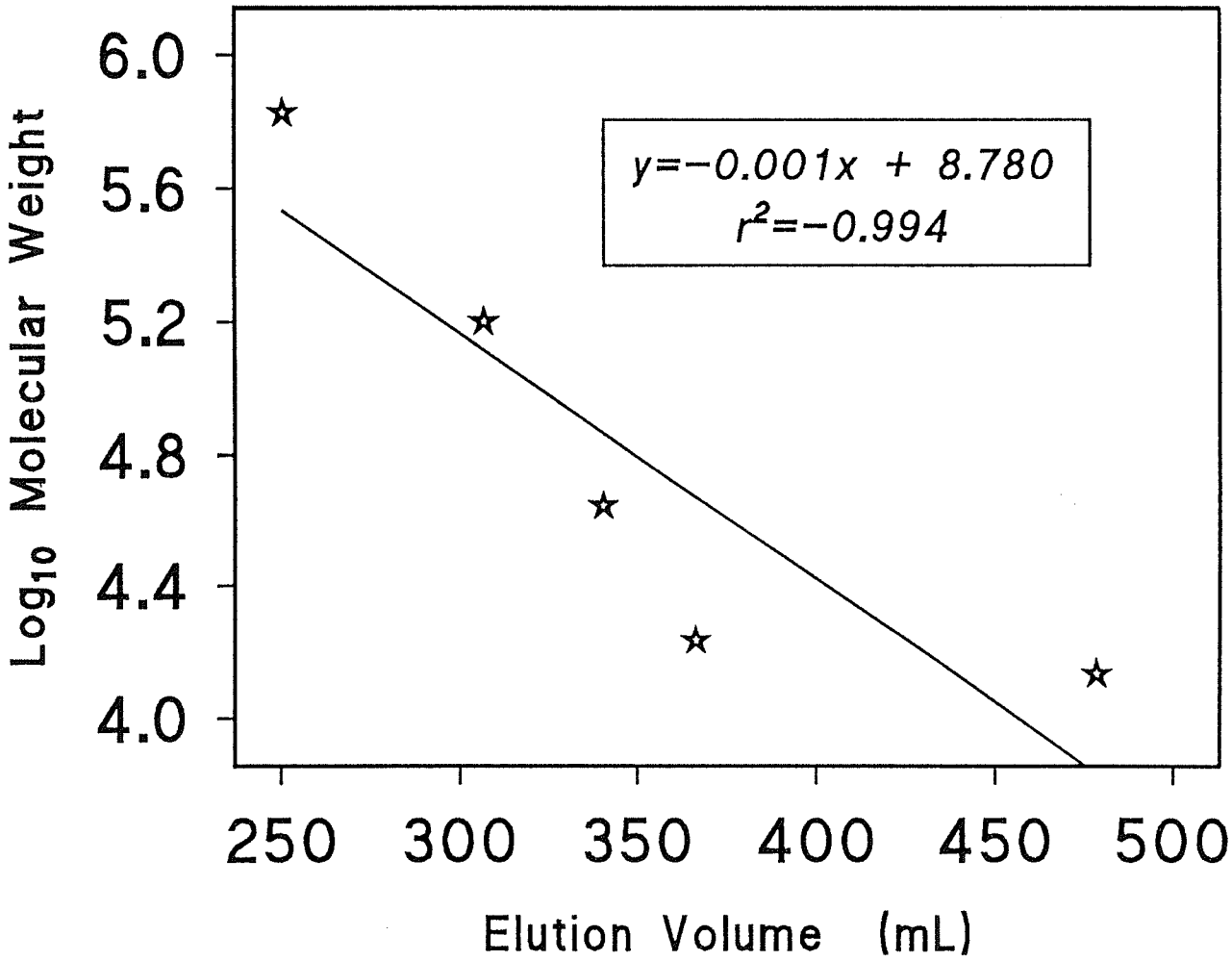
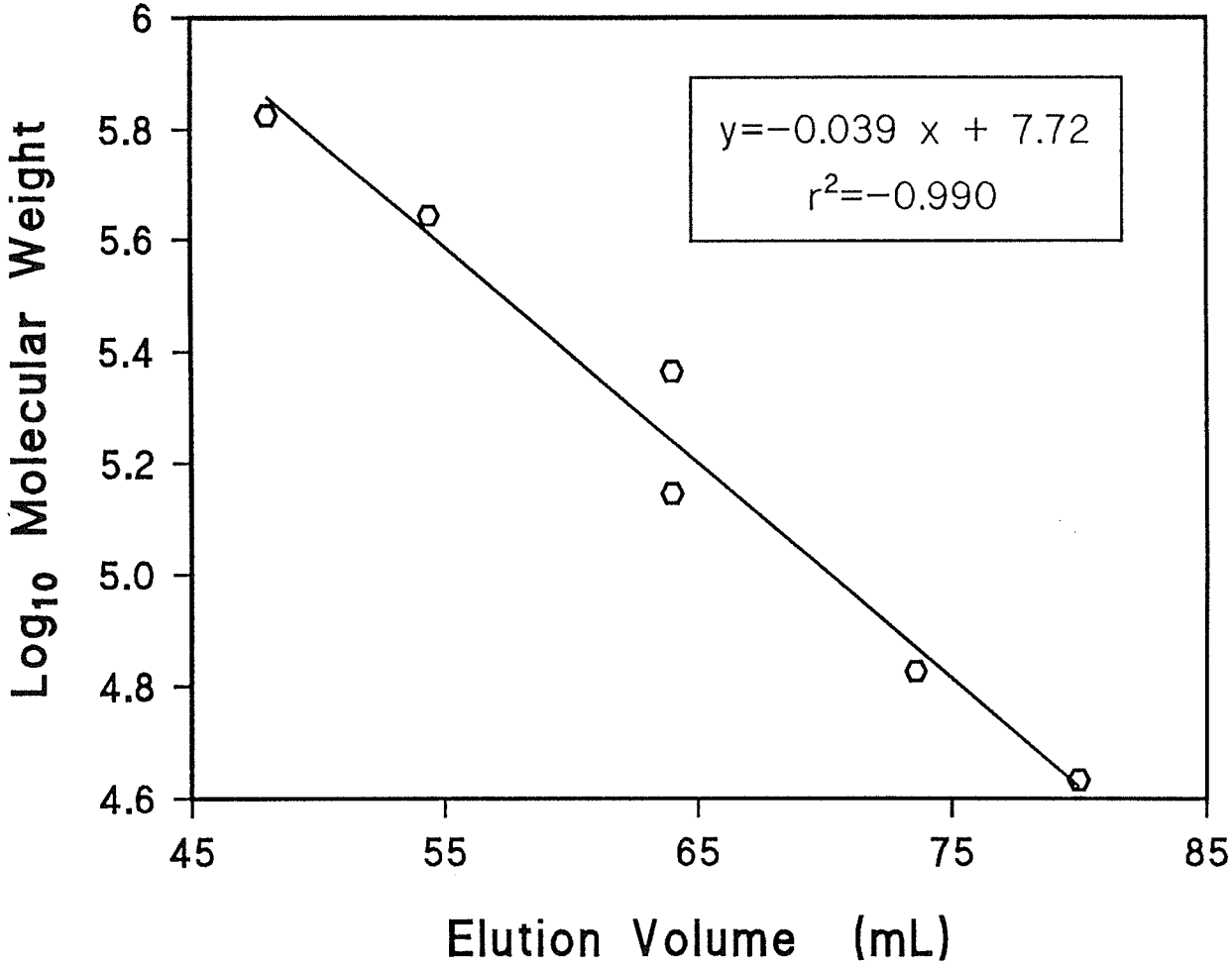
Appendix A. A sample calibration curve for molecular weight determination of the major salt soluble canola protein using Sephacryl S-300 column equilibrated with 0.5M NaCl with 0.2% sodium azide (w/v)


6.0
5.6
5.2
4.8
4.4
4.0
Log10 Molecular Weight
Y=-0.002x + 9.89
r2=-0.994
250
300
350
400
450
500
Elution Volume (mL)

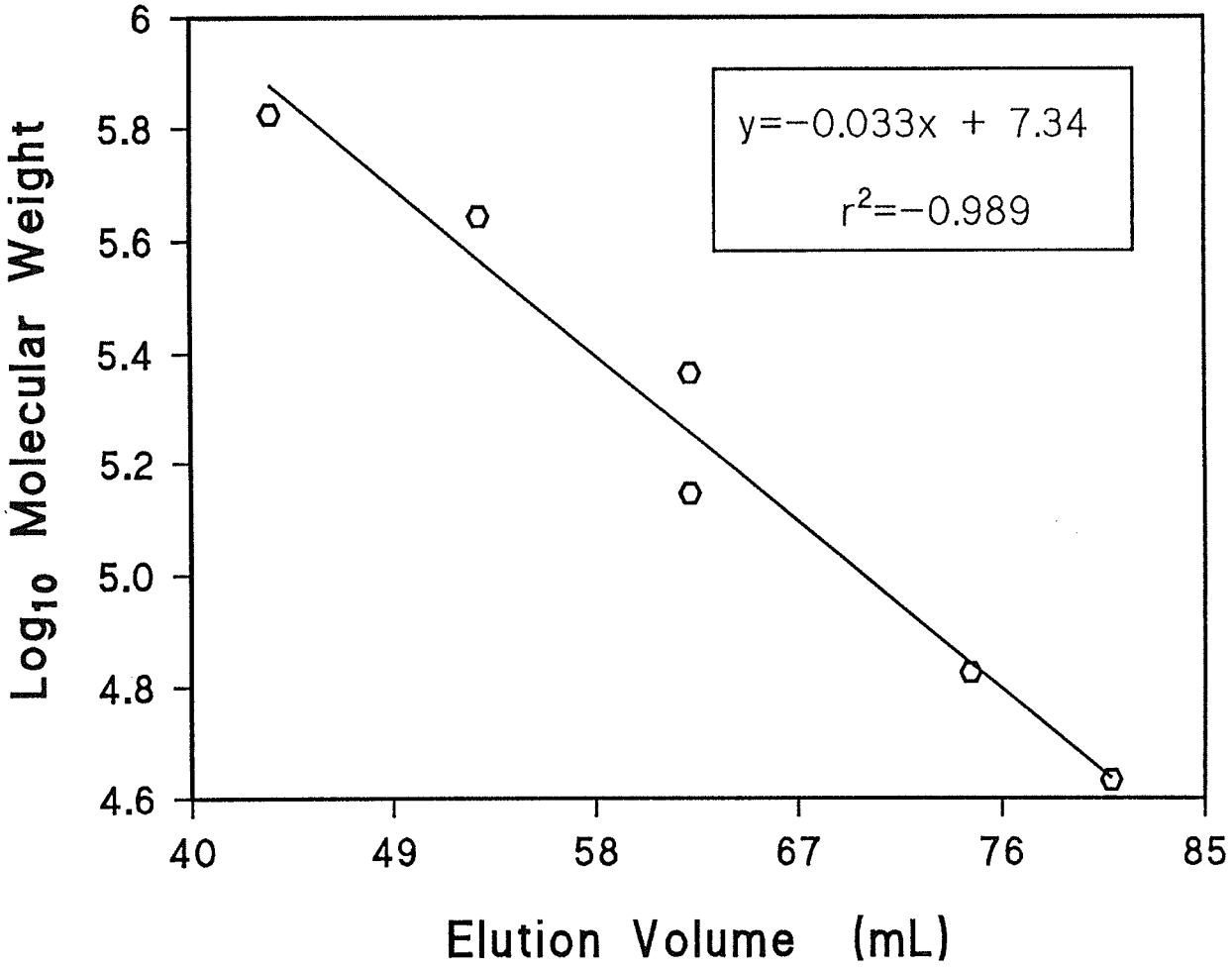
Appendix B. A sample calibration curve for molecular weight determination of the major salt soluble canola protein using Sephacryl S-300 column equilibrated with 2.0M NaCl with 0.2% sodium azide (w/v)


6.0
5.6
5.2
4.8
4.4
4.0
Log10 Molecular Weight
y=−0.001x + 8.780
r2=−0.994
250
300
350
400
450
500
Elution Volume (mL)

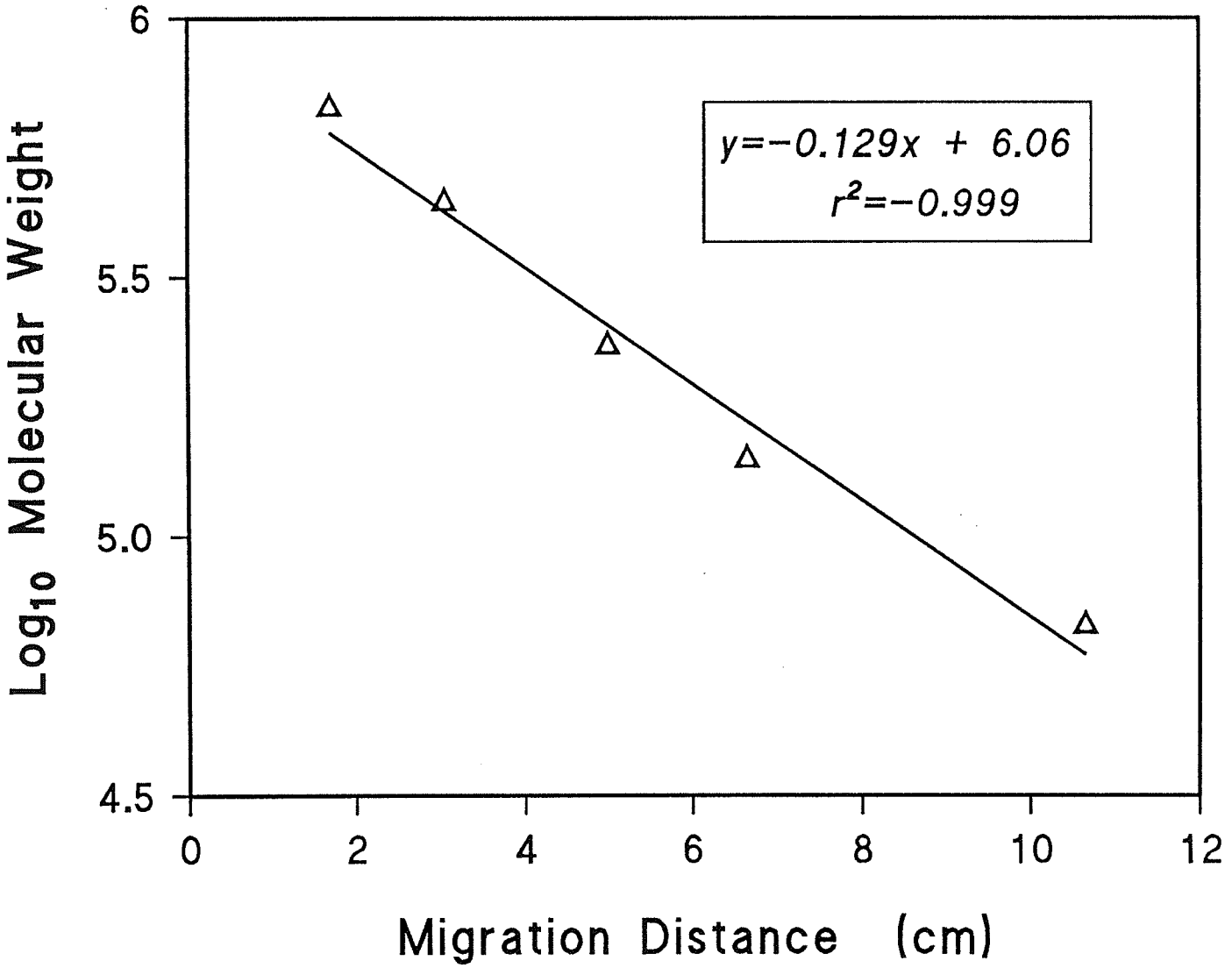
Appendix C. Calibration curve for molecular weight determination of the 12S canola globulin using a HiLoad 16/60 Superdex 200 prep grade gel filtration column equilibrated with 0.5M NaCl pH 6.0


y=−0.039 x + 7.72
r²=−0.990
Log10 Molecular Weight
6
5.8
5.6
5.4
5.2
5.0
4.8
4.6
45
55
65
75
85
Elution Volume (mL)

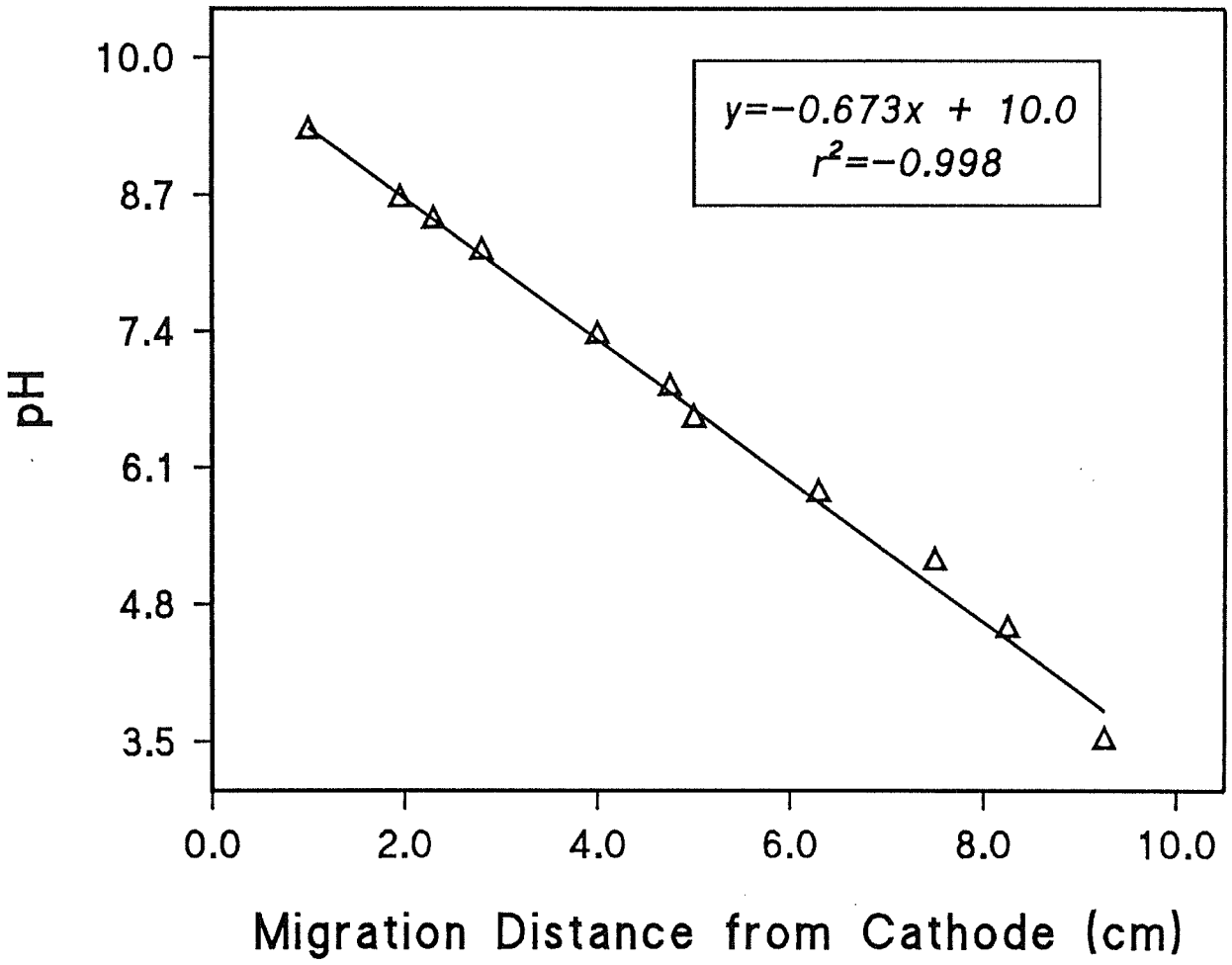
Appendix D. Calibration curve for molecular weight determination of the 12S canola globulin using a HiLoad 16/60 Superdex 200 prep grade gel filtration column equilibrated with 0.5M NaCl pH 4.0


y=−0.033x + 7.34
r²=−0.989
Log₁₀ Molecular Weight
6
5.8
5.6
5.4
5.2
5.0
4.8
4.6
40
49
58
67
76
85
Elution Volume (mL)

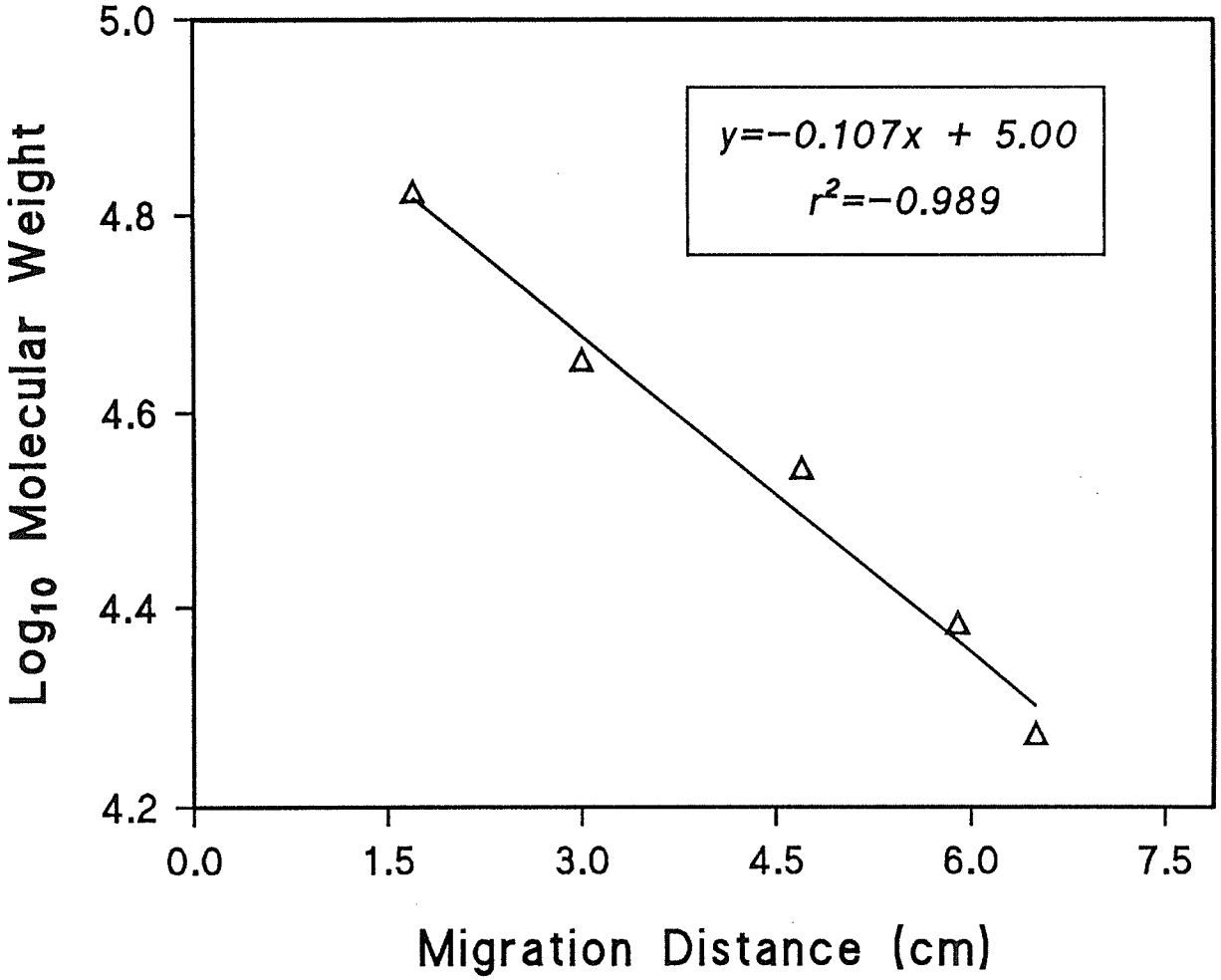
Appendix E. Calibration curve for molecular weight determination of the 12S canola globulin using gradient electrophoresis


6
5.5
5.0
4.5
0
2
4
6
8
10
12
Log10 Molecular Weight
Migration Distance (cm)
y=−0.129x + 6.06
r²=−0.999

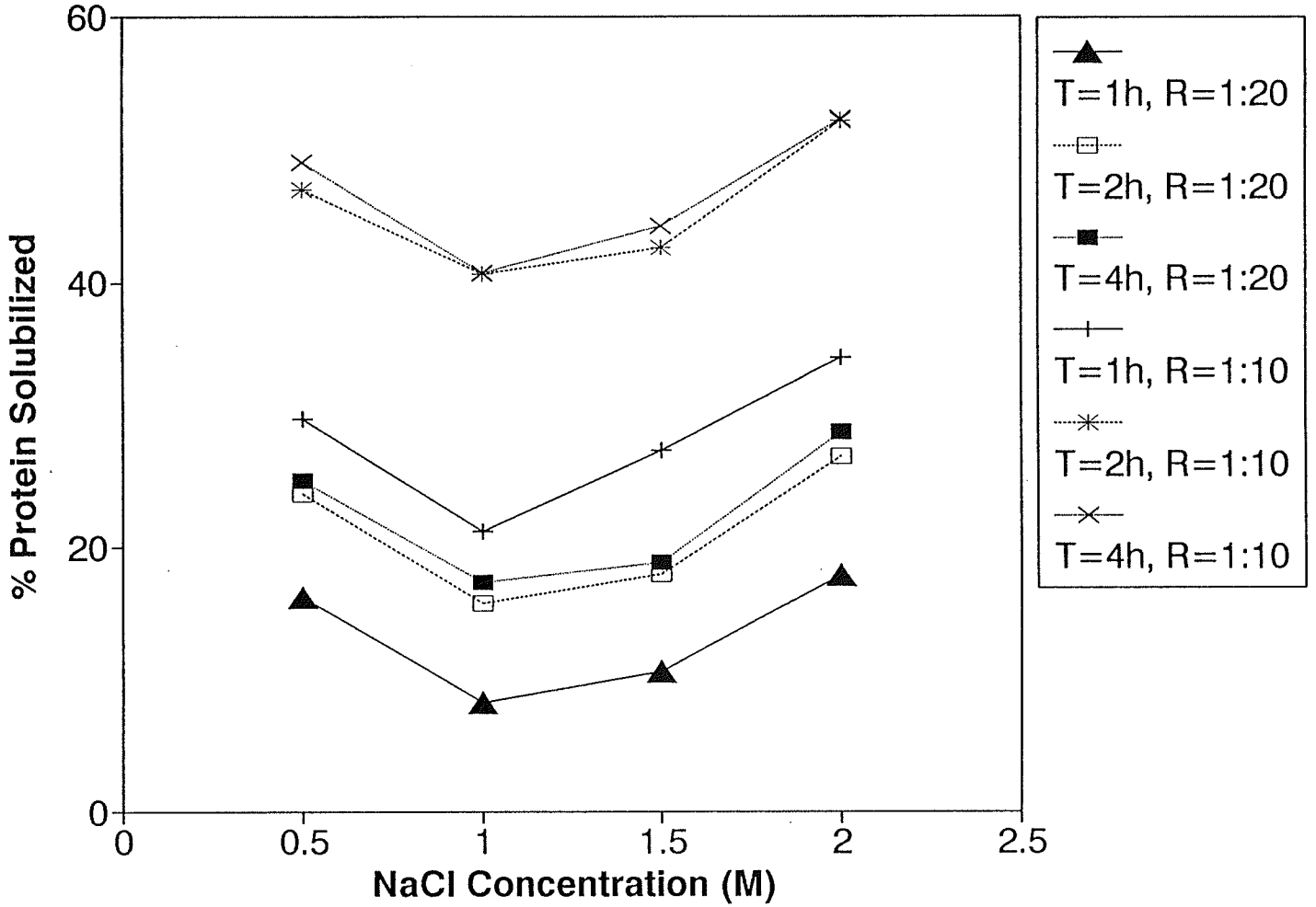
Appendix F. Calibration curve for isoelectric point determination of the 12S canola globulin by isoelectric focusing


y=−0.673x + 10.0
r²=−0.998
10.0
8.7
7.4
6.1
4.8
3.5
pH
0.0
2.0
4.0
6.0
8.0
10.0
Migration Distance from Cathode (cm)

Appendix G. Migration distance of the SDS-PAGE protein standards as a function of $\log_{10}$ molecular weight


y=−0.107x + 5.00
r²=−0.989
Log10 Molecular Weight
5.0
4.8
4.6
4.4
4.2
0.0
1.5
3.0
4.5
6.0
7.5
Migration Distance (cm)

Appendix H. The effect of time and meal to solvent ratio on nitrogen extractability of Westar canola meal


60
40
20
0
% Protein Solubilized
0
0.5
1
1.5
2
2.5
NaCl Concentration (M)
T=1h, R=1:20
T=2h, R=1:20
T=4h, R=1:20
T=1h, R=1:10
T=2h, R=1:10
T=4h, R=1:10

Appendix I. Effect of NaCl molar concentration on nitrogen extractability of Westar canola meal

NaCl Molar Concentation	Ionic Strength (ms/cm)	pH		Protein Recovery
		Before	After	
0.05	5.49	5.899	5.9213	22.58±2.49[a]
0.10	10.2	5.899	5.909	30.45±3.76[ab]
0.25	24.2	5.892	5.917	34.97±0.59[abc]
0.50	42.7	5.906	5.924	47.03±0.83[de]
0.75	65.5	5.925	5.90	38.48±8.10[bcd]
1.00	80.6	5.930	5.946	40.55±2.16[bcd]
1.25	97.9	5.919	5.941	43.98±2.32[cde]
1.50	113.3	5.920	5.919	42.60±3.48[cd]
1.75	130.0	5.908	5.923	44.97±7.16[cde]
2.00	143.0	5.890	5.908	52.19±1.04[e]

[a] All extraction were preformed at room temperature, 1:10 protein to solvent ratio, and mixed for 2h.

Appendix J. Effect of SHMP concentration (%) on nitrogen extractability of Westar canola meal

SHMP (%)	Ionic Strength (ms/cm)	pH		Protein Recovery
		Before	After	
0.01	0.96	5.980	5.983	26.40±2.55[a]
0.05	0.37	6.004	5.999	27.07±1.34[ab]
0.10	0.94	6.032	6.049	31.35±2.25[ab]
0.25	1.05	6.118	6.118	34.82±3.69[b]
0.50	2.44	6.175	6.178	51.97±3.70[c]
1.00	4.20	6.289	6.229	52.87±2.29[c]
1.50	5.98	6.270	6.250	54.35±2.41[c]
2.00	6.91	6.235	6.225	50.70±6.22[c]
3.00	9.61	6.179	6.108	54.45±10.11[c]

[a] All extractions were preformed at room temperature, 1:10 protein to solvent ratios and mixed for 2h.

Appendix K. The effect of pH and salt concentration on the thermal properties of the major salt soluble canola proteins

A.

NaCl Molar Concentration

pH	0.1	0.3	0.5
	-*	-*	11.68±0.97 [2]
6	21.05±8.05[a1]	20.37±2.75[a1]	20.88±0.40[a2]
7	23.57±2.88[a1]	23.17±3.41[a1]	22.74±0.48[a2]
8	25.67±2.11[b1]	22.86±1.47[a1]	23.25±0.32[a2]
9	27.59±2.13 [1]	-*	-*

Means±SD values within each group followed by the same letter (in row; salt effect) or numeral (in column; pH effect) are not significantly different (P≤0.05).

B.

NaCl Molar Concentration

pH	0.1	0.3	0.5
4	-*	-*	82.30±0.73[1]
6	83.03±0.77[a1]	89.43±0.50[b1]	91.43±0.30[c2]
7	86.45±0.16[a2]	90.68±0.44[b2]	92.49±0.48[c3]
8	89.44±0.68[a3]	92.41±0.59[b3]	93.20±0.07[c4]
9	91.30±0.27[a4]	-*	-*

Means±SD values within each group followed by the same letter (in row; salt effect) or numeral (in column; pH effect) are not significantly different (P≤0.05).

* The Td and ΔH values for these environments were not experimentally determined.