EFFECTS OF ACETYLATION AND SUCCINYLATION ON THE PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF THE CANOLA 12S GLOBULIN

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BY

LISA GRUENER

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

MASTER OF SCIENCE

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DEDICATION

This thesis is specially dedicated to my parents for their unending love and support, which has given me the confidence and determination to pursue my goals.

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ABSTRACT

The canola 12S globulin was isolated by the protein micellar mass (PMM) procedure and acylated through acetylation and succinylation in order to obtain a canola protein concentrate with optimal functional properties and low levels of antinutritional factors. Changes in molecular weight were determined by gradient PAGE. Alterations in protein conformation were examined by differential scanning calorimetry (DSC), UV/vis and fluorescence spectroscopy. The isoelectric points of the PMM and acylated concentrates were established by isoelectric focusing. In addition, changes in surface hydrophobicity, amino acid profile and levels of antunutritional factors were also monitored. Several functional properties of the PMM and acylated concentrates, specifically protein solubility, foaming and emulsifying properties, fat and water absorption, colour and gelation were also studied.

Acylation was found to cause dissociation of the protein structure, which was evidenced by a decrease in molecular weight and enthalpy of denaturation with increasing modification. The isoelectric points of the acylated concentrates differed from the value of 7.1 observed in the PMM with ranges of 4.9-6.1 for the acylated concentrates, and 4.6-5.5 for the succinylated concentrates. Extensive changes in protein conformation were also indicated by alterations in the UV/vis and fluorescence spectra, as well as changes in surface hydrophobicity. Generally, these structural changes were more pronounced in the succinylated concentrates as compared to the acetylated concentrates. There was a slight decrease in lysine

following acylation and proline following succinylation, but otherwise the amino acid profile remained unchanged. Both acetylation and succinylation resulted in an overall decrease in the levels of phytic acid and glucosinolates. Acetylation also caused a decrease in the phenolics content.

The solubility profile of the acylated concentrates differed from that of the PMM in that solubility below the isoelectric point was impaired, but solubility at neutral and alkaline pH values was greatly enhanced. Foaming and emulsifying capacities were significantly increased by acylation. Foam stability declined upon acylation. Emulsion stability increased initially, then decreased at the highest levels of modification. Following acylation, the water holding capacity was reduced dramatically, but the fat absorption capacity was elevated. Gelling properties of canola proteins were also improved by acylation. Furthermore, the acylated concentrates were significantly lighter in colour than the original PMM. Overall, the acylated concentrates possessed improved functionality and lower levels of antinutritional factors as compared to the PMM, making them more suitable as a food or cosmetic ingredient.

INTRODUCTION

The cost of food grade proteins has increased dramatically, along with a trend towards complete food formulations from refined ingredients (Franzen and Kinsella, 1976a). Therefore, there is a growing need for less expensive protein, such as those available from plant sources. In addition, current marketing trends in the cosmetic industry are directed towards minimizing the use of animal-derived ingredients. In order to be incorporated into food and cosmetic products, these plant proteins must possess the desired functional properties. Limited functionality is one of the main barriers restricting the use of plant proteins in the industry today. However, proteins may be modified through chemical or enzymatic means in order to improve their functional and sensory properties, or to remove undesirable factors (Kinsella, 1976).

Chemical modification by acylation has been used as a tool for improving the properties of a large number of plant proteins (Franzen and Kinsella, 1976b; Canella *et al.*, 1979; Ma *et al.*, 1986; Ponnampalam *et al.*, 1990). Acylation is a desirable form of modification as it is performed under relatively mild reaction conditions, is fairly inexpensive, and has a high reaction specificity (Ball and Winn, 1982).

Canola, the dominant oilseed crop grown in Canada today, is a valuable source of protein with a well-balanced amino acid composition and a high content of sulphur-containing amino acids (Ohlson and Anjou, 1979). However, its use in food and cosmetic products is limited due to inferior functionality as compared to other

commercially available protein isolates, and the presence of undesirable antinutritional factors.

The main objective of this study was to obtain a canola protein concentrate with improved functional properties. The canola 12S globulin was isolated from defatted canola meal by the protein micellar mass (PMM) procedure. The PMM was then modified through acetylation and succinylation to obtain eight protein concentrates with varying levels of acylation. Following acylation, several physicochemical characteristics of the PMM and acylated concentrates were examined, including factors such as molecular weight, surface hydrophobicity and thermal properties. Several functional properties, such as protein solubility, foaming and emulsifying properties, and gelation, were studied. In addition, changes in the levels of phytic acid, phenolics, and glucosinolates were also monitored.

LITERATURE REVIEW

A. The Canola Protein

1. Protein content and composition

Canola is the dominant oilseed crop grown in Canada. The mature seed contains approximately 24% protein; however, the protein content may vary from 11-42% depending upon genetic and environmental factors (Mieth *et al.*, 1983). The remainder of the seed consists of 40% oil, 20% carbohydrate, and miscellaneous other low molecular weight substances (Bhatty *et al.*, 1968).

There are three protein fractions present in canola meal. These fractions may be separated and characterized according not only to their solubility and precipitability; but also on the basis of differing ultracentrifugal, chromatographic and electrophoretic behaviour (Mieth *et al.*, 1983). The largest of these fractions is the salt-soluble globulins or storage proteins. The function of these storage proteins is to serve as nitrogen reserves for the embryonic axis during germination (Norton, 1989). The water-soluble albumins contain the majority of the metabolically active proteins, and are responsible for the biosynthesis and degradation of the globulins. The third protein fraction found in canola are the alkali soluble proteins (Norton, 1989).

According to Mackenzie and Blakely (1972), the predominant protein in *Brassica* spp. is the 12S globulin (cruciferin). The 12S globulin is a high molecular weight neutral complex composed of several polypeptide chains. Three other protein fractions, the 1.7S, 7S, and 15S are also present. The 1.7S globulin (napin) is the

second largest fraction and is a low molecular weight basic protein consisting of two disulfide-linked polypeptide chains (Ericson *et al.*, 1986). Norton (1989) suggested that the 12S globulin accounted for 60% of the total seed proteins, and the 1.7S globulin approximately 20%. In *Brassica* spp., particularly rapeseed varieties, the 7S fraction is very low or nonexistent (Prakash and Rao, 1986). Mackenzie (1975) reported that the amino acid content of the 15S fraction was indistinguishable from the 12S fraction, indicating it was probably a dimer of the 12S protein, rather than being inherently present in the seed.

2. Physicochemical Properties of the 12S Globulin

a. Molecular Weight and Subunit Profile.

Several studies on the fractionation and isolation of the canola 12S globulin have been performed. Bhatty *et al.* (1968) was the first to extract the 12S globulin from defatted rapeseed meal by extraction with 10% sodium chloride, precipitation by dialysis against water, and chromatographic separation on Sephadex G-100. Simard and Boulet (1978) isolated the protein by fractionation, precipitation and dissolution by ammonium sulphate. Schwenke *et al.* (1981) applied a combined gel filtration and ion-exchange chromatography method to purify the 12S globulin.

The 12S globulin from canola is similar to the other 12S globulins of various oilseeds, with a molecular weight of approximately 300,000 and an oligomeric structure (Reichelt *et al.*, 1980). A summary of the physicochemical properties of the 12S globulin is given in Table 1.

TABLE 1. Physicochemical properties of the 12S globulin from rapeseed[1]

PROPERTY	VALUE
Molar Mass (g/mol)	300,000
Isoelectric Point	7.2
Sedimentation Coefficient $S^{\circ}_{20,w}[10^{-13}sec]$	12.7
Diffusion Coefficient $D^{\circ}_{20,w}[10^{-7}m^2/sec]$	3.8
Stokes Radius (Rs, nm)	
Quasielectric light scattering	5.7
Gel chromatography	5.5
Partial Specific Volume (mL/g)	0.729
Frictional Ratio (f/fo)	1.28
Molecular Weight	
Sedimentation velocity and diffusion	300,000
Sedimentation velocity and gel chromatography	294,000
Dimension (nm)	
Electron microscopy	.3 x 11.3 x 9.2
Small angle scattering	10.5 x 10.5 x 9.2

TABLE 1. (Cont'd)

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

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

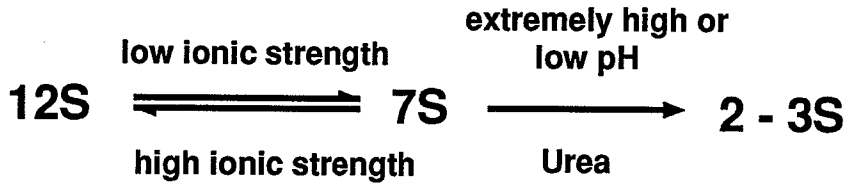
The 12S globulin consists of six polypeptide chains, with each subunit made up of two polypeptide chains which are linked by disulfide bonds (Norton, 1989). According to Schwenke *et al.* (1983), each of the smaller polypeptide chains (PPC1 and PPC2) combined with the larger PPC3 or PPC4 to form one monomeric subunit with a molecular weight of approximately 50,000. Reichelt *et al.* (1980), studied the quaternary structure of the 12S globulin using electron microscopy, and proposed that the subunits were arranged in the form of a trigonal antiprism with a dihedral point group symmetry of 32.

b. Dissociation Profile

According to Bhatty *et al.* (1968), the rapeseed protein is present as a hexameric native 12S globulin in high ionic strength solutions. The globulin's quaternary structure undergoes a stepwise dissociation depending on the protein's environment. This stepwise dissociation of the 12S globulin is illustrated in Figure 1.

When the ionic strength is less than or equal to 0.5, the 12S globulin undergoes a reversible dissociation to the 7S trimeric intermediate (Schwenke *et al.*, 1981). In the presence of strong dissociating agents (4-6 M urea) or pH extremes, especially acidic conditions below pH 3.6, the 7S proteins irreversibly dissociate to the 2-3S monomeric form (Goding *et al.*, 1970). The 2-3S monomers may in turn dissociate into their acidic and basic subunits in the presence of reducing agents such as mercaptoethanol (Schwenke *et al.*, 1983; Mieth *et al.*, 1983).

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



3. Antinutritional Factors in Canola

a. Phytic Acid

Phytates, the mixed calcium and magnesium salts of phytic acid, are present in many grains and oilseeds. The typical phytic acid content of defatted canola meal is approximately 3.69% on a dry weight basis (Erdman, 1979). Phytic acid binds divalent minerals such as Zn, Cu, Ca, Fe, Mn, Mo and Co (Nolan *et al.*, 1987; Miller *et al.*, 1986; Bjorck and Nyman, 1987). They may also interact with proteins, forming complexes which cannot undergo enzymic digestion (Cheryan, 1980). Cheryan (1980) reported that the interaction of phytic acid with protein was a result of charge attractions, and that these interactions are recognized at 3 pH regions: low, intermediate and high. At low pH levels, below the isoelectric point, the positively charged protein complexes with the negatively charged phytic acid to form an unionized salt. At intermediate pH levels, the phytic acid reacts with ions and proteins to form a ternary phytic acid-cation-protein complex, the stability of which increases up to a pH of 10. Beyond pH 10, the mineral-phytate-protein complex is disrupted and the phytic acid becomes insoluble (Cheryan, 1980).

b. Phenolic Compounds

Many plants contain phenolic acids and their various derivatives. Phenolics contribute to the dark colour, bitter taste and astringency of canola products (Kozlowska *et al.*, 1983). In addition, their oxidized products may form complexes with essential amino acids, enzymes and other proteins, rendering them unable to become assimilated in the digestive tract. Ponnampalam *et al.* (1987) reported that phenolics negatively impacted the functional properties of rapeseed protein.

Rapeseed has been found to contain a wide variety of phenolic acids (Kozlowska *et al.*, 1983). They are present in three forms: free, esterified and bound. The esterified portion contains up to 80% of the total phenolics. Sinapine, the choline ester of sinapic acid, constitutes 80-99% of the total amount of esterified phenolic compounds in rapeseed (Kozlowska *et al.*, 1983; Ponnampalam *et al.*, 1987). Other phenolic compounds include p-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, p-coumaric and ferulic acids.

c. Glucosinolates

Glucosinolates may be found in all plant foods of the family *Cruciferae*; however, the type and the level vary among different plant species (Mieth *et al.*, 1983). They appear to be the most limiting factor for the use of canola proteins for human consumption, as they are hydrolysed by the enzyme myrosinase to yield decomposition products including isothiocyanates, thiocyanates and nitriles (Appelqvist and Ohlson, 1972).

The primary antinutritional effect of the glucosinolates are their disruptive activities upon basic metabolism by negatively influencing iodine metabolism (Mieth *et al.*, 1983). Appelqvist and Ohlson (1972) determined that ingestion of rapeseed caused thyroid enlargement by interfering with thyroxin synthesis. In addition, nitriles have been shown to cause enlargement of the liver and kidneys coupled with lesions in both organs (Van Etten, 1969).

B. Isolation of Proteins Using Micelles

Murray *et al.* (1981) developed a procedure for processing and utilizing plant proteins known as the protein micellar mass (PMM) method. This was a noncovalent approach to protein isolation based on the premise that when amphiphilic molecules are dissolved in water they can achieve segregation of their hydrophobic portion from the solvent by self-aggregation into formations called micelles (Tanford, 1973). This is a mild technique which involves solubilization of the protein in a high salt environment followed by a rapid reduction of ionic strength to precipitate the protein in the form of insoluble protein micelles.

Using the protein micellar mass method of Murray *et al.* (1981), Arntfield *et al.* (1985) were able to recover 42.5% of the protein from fababean. Welsh (1988) later applied this method to the isolation of canola proteins, but recovered less than 5% of the protein. By manipulation of the extraction environment and dilution factors in the PMM procedure, Burgess (1991) recovered approximately 31% of the protein from canola.

Subsequently, Ismond and Welsh (1992) used the PMM procedure to isolate the canola globular protein, concentrating on the removal of antinutritional compounds. They concluded that a 0.1M NaCl/0.1M Na_2HPO_4 buffer at pH 5.5 was optimal for the removal of phytic acid and phenolic compounds. Maximum removal of glucosinolates was obtained in a 0.01M NaCl/0.01M Na_2HPO_4 buffer system at pH 5.5.

C. Modification of Proteins

Modification of plant proteins to impart them with desirable functional properties would aid in meeting world protein requirements. Protein functionality is determined by several factors, including the protein's intrinsic physicochemical characteristics, interactions with protein and other nonprotein components, and environmental conditions of the food system (Kester and Richardson, 1984). The physicochemical characteristics of a protein include properties such as molecular weight, amino acid composition and sequence, net charge and surface hydrophobicity. Therefore, protein modification involves the intentional alteration of one or more of these properties resulting in a change in the functionality (Kester and Richardson, 1984). Improved functionality may not be the only objective in modifying a protein; an improvement in flavour, colour and destruction of undesirable enzymes, antinutritive factors, hemagglutinins and allergens may also be considerations (Kinsella, 1977).

The most common type of modification performed on proteins is hydrolysis by acid, alkali, or enzymes to form mixtures of smaller peptides; however, there are many problems associated with this procedure (Franzen and Kinsella, 1976a). With acid hydrolysis, excessive hydrolysis and humin formation may occur. Alkaline hydrolysis may cause racemization of amino acids, formation of potentially toxic compounds, lysinoalanine formation, and cross-linking of proteins making them more resistant to digestion (Franzen and Kinsella, 1976a). Lastly, enzymic hydrolysis may result in the formation of bitter peptides.

An alternate method, chemical modification, entails derivitization of certain amino acid side chains in the protein. This causes an alteration in the noncovalent forces such as van der Waals, electrostatic interactions, hydrophobic interactions and hydrogen bonding, which influence protein conformation (Kester and Richardson. 1984). This in turn results in changes in the physicochemical and functional properties of the proteins. There are many types of chemical derivitizations. Of these, acylations with acetic or succinic anhydride are two possible methods.

1. Acetylation and Succinylation

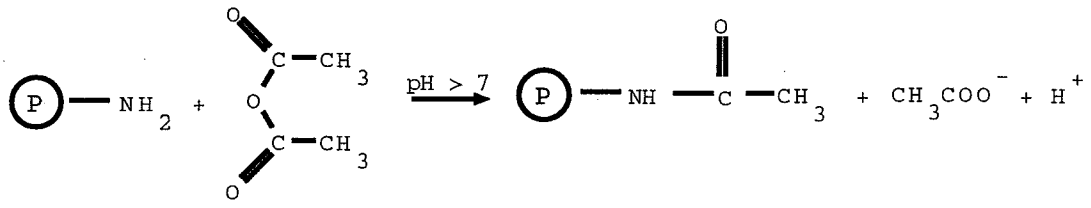
Acylation is a commonly performed method of chemical modification for many reasons. First of all, the amino groups tend to be located on the surface of the protein molecules, and are therefore easily accessible for attachment by the acylating reagents (Riordan and Vallee, 1971). Both acetylation and succinylation are reactions which are rapid, highly specific, and are performed under mild conditions. Another benefit is that the degree of acylation of a protein may be determined by simple analytical procedures, such as measuring the decrease in the number of amino groups known to be present in the protein (Riordan and Vallee, 1971).

The acetyl and succinyl groups may attach to all nucleophilic groups of amino acid residues, including the ε-amino groups of lysine, the hydroxyl groups of serine, the phenol groups of tyrosine, and the imidazole groups of histidine (Franzen and Kinsella, 1976a). Of these, the lysine ε-amino group reacts more readily because of a relatively low pK. As well, this group is hydrophilic in nature and

projects from the molecular surface into the aqueous media, lending it steric availability to contact with the acylating reagent (Tanford, 1973).

In acetylation, a neutral acetyl group is attached to the protein molecule (Ball and Winn, 1982). The mechanism of acetylation is illustrated in Figure 2. The addition of the neutrally charged acetyl groups to the positively charged ε-amino groups results in changes in the net charge. Overall, the net surface charge becomes more negative relative to the ionic properties of the native protein (Ball and Winn, 1982).

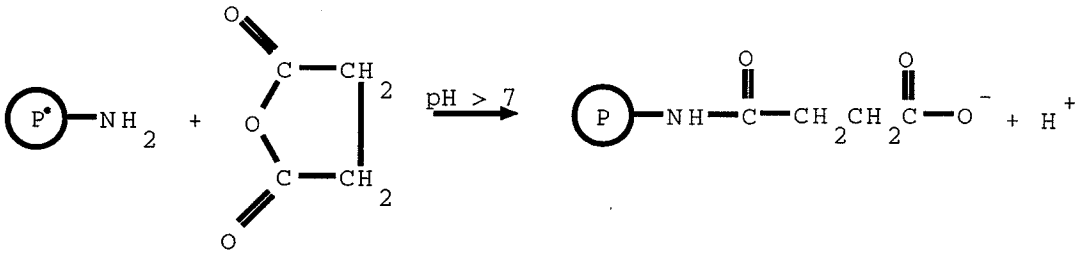
Figure 2. Mechanism of acetylation (Means and Feeney, 1971)



Succinylation introduces a negatively charged bulky succinyl group into the protein structure. This has three major effects on the physical character of the protein. These include increasing the net negative charge (Habeeb *et al.*, 1958); changing the conformation (Hass, 1964); and increasing the dissociation of the protein into subunits (Klotz and Kereseztes-Nagy, 1962). Dissociation is due to the high net negative charge and the replacement of short range attractive forces in the native molecule with short range repulsive ones, followed by unfolding of polypeptide chains (Franzen and Kinsella, 1976a). The mechanism of succinylation is illustrated in Figure 3. In acetylated proteins, these structural changes are less

pronounced, as the acetyl group results in fewer electrostatic attractions, and does not produce repulsive forces.

Figure 3. Mechanism of succinylation (Means and Feeney, 1971)



2) Impact of Acylation on Functional Properties

The functional properties of a protein refer to its intrinsic physicochemical characteristics which affect the behaviour of protein in food systems during processing, storage and preparation (Kinsella, 1979). There are many categories of functional properties, including: organoleptic, kinesthetic (flavour, odour, colour, texture); hydration, dispersibility, solubility, swelling, rheological, surface active properties (emulsifying, foaming) and gelation (Kinsella, 1977).

One of the most important functional properties of a food protein is solubility, such as in beverages (Snyder and Kwon, 1987). Solubility is also important for ease of distribution when using a food protein as a gelling, emulsifying or foaming agent (Snyder and Kwon, 1987). In their native state, most food proteins are only sparingly soluble. Acylation has been found to markedly improve the solubility of many types of plant proteins, including wheat gluten (Barber and Wartheson, 1982), peanut protein (Beuchat, 1977), cottonseed protein (Childs and Park, 1976), sunflower protein (Canella *et al.*, 1979; Kabirullah and Wills, 1982), soy protein

(Franzen and Kinsella, 1976a) and canola protein isolates (Paulson and Tung, 1987). In most cases, solubility was greatly increased at neutral to slightly alkaline pH levels. Acylation has been shown to impair solubilization below the isoelectric point. Generally, succinylation was far more effective in improving solubility than acetylation.

The ability of a protein to lower interfacial tension and form a film determines its effectiveness as a foaming or emulsifying agent. When used as emulsifiers, food proteins have many functions, including fat-micelle stabilization, viscosity control, and texture control in a variety of products, such as comminuted meats, coffee whiteners, mayonnaise, salad dressings and frozen desserts. Several studies have reported an improvement in both foaming and emulsifying properties upon succinylation (Canella *et al.*, 1979; Childs and Park, 1976; Franzen and Kinsella, 1976b; and Gueguen *et al.*, 1990). Acetylation has also been found to improve foaming and emulsifying properties, but to a lesser extent than succinylation. However, both succinylation and acetylation have been found to decrease foam stability (Ma and Wood, 1987; Narayana and Rao, 1984; Rahma and Rao, 1983).

Gelation is another important functional property of food proteins. Protein gels provide a structural matrix for holding water, sugars and food ingredients in a variety of food products such as comminuted meats, meat analogs and cheeses (Kinsella, 1979). Both succinylation and acetylation were found to improve the gelation properties of oat protein (Ma and Wood, 1987). In particular, succinylation

improved the gelation properties of canola protein isolates (Paulson and Tung, 1989) and cottonseed flour (Choi *et al.*, 1982).

Fat and water adsorption are responsible for many functions in food products. Water holding properties of food proteins are necessary in a variety of meats, bakery products and cheeses, while fat adsorption is extremely important in comminuted meats (Kinsella, 1979). Studies performed on cottonseed flour (Childs and Park, 1976), on sunflower proteins (Kabirullah and Wills, 1982) and on winged bean flour (Narayana and Rao, 1984) indicate that acetylation is successful in improving the water and fat adsorption properties of these proteins. However, studies by Ma and Wood (1987) plus Ma (1984) reported a decrease in water adsorption following acylation, which was attributed to the increased solubility of the protein.

3. Nutritional Considerations

Chemical modification is an excellent method of improving protein functionality. Optimally, the modification procedure should not cause deterioration in the nutritional quality of a protein. However, Kinsella (1977) stated that in improving the functional properties of a protein, loss of some essential amino acids may be inevitable.

Canella *et al.* (1979) reported no significant changes in the amino acid composition other than lysine following acylation of sunflower proteins. Franzen and Kinsella (1976a) reported the same results with soy protein isolates, as did Ponnamaplam *et al.* (1990) with rapeseed. However, Franzen and Kinsella (1976b)

noticed no apparent destruction of amino acids in leaf protein due to acetylation or succinylation. In a study on oat proteins, succinylation caused a decrease in phenylalanine, but no other essential amino acids. Acetylation resulted in a slight decrease in cystine (Ma and Wood, 1987).

The *in vitro* digestibility of pea protein isolates was not impaired by acylation (Johnson and Brekke, 1983). The same results were reported by Ponnampalam *et al.* (1990) on rapeseed.

The nutritional quality of acylated rapeseed proteins was further investigated by Ponnampalam *et al.* (1990). Succinylation was found to significantly lower the net protein ratio (NPR), apparent digestibility coefficient (ADC) of nitrogen and weight gain. However, supplementation with lysine significantly improved the NPR, weight gain, feed intake, and ADC of nitrogen.

These decreases in nutritional value were all observed at high levels of acylation. Kinsella (1977) stated that to obtain significant increases in functional properties, extensive acylation was not necessary; therefore the detrimental effects of acylation on the nutritional value of the protein may not be a concern.

4. Non-food Applications for Modified Proteins

Canola proteins may also have potential as a non-food component, such as an additive in cosmetic systems. Cosmetic proteins provide subjective benefits, such as improvements in aesthetic attributes of both hair and skin, as well as technical benefits through their influence on the characteristics and physical properties of the

formulations into which they are incorporated (Teglia *et al.*, 1993).

The most important functional property of cosmetic proteins is substantivity. Substantivity refers specifically to the number of peptides not extracted by water due to the formation of weaker or stronger linkages with the skin or hair (Teglia *et al.*, 1993). Mintz *et al.* (1991) reported that there were two distinct groups of proteins which bound to hair, a high molecular weight group of greater than 30,000, and a low molecular weight group of 1,000 to 3,000. Subsequent research by Gallagher and Jones (1992) showed that the high molecular weight group between 30,000 and 300,000 provided the most dramatic subjective conditioning benefits for hair due to their film-forming properties.

As with food systems, foaming and emulsifying properties are extremely important in cosmetic systems. Foam is a dominant factor which determines the acceptability of many cosmetic products such as soap, shampoo, shaving foam and toothpaste (Rieger, 1991). However, while foaming capacity is important, cosmetic foams are not required to be stable for long periods of time. Cosmetic proteins with good emulsifying properties may function as the primary emulsifier or coemulsifier in creams and lotions (Scafidi and Neghme, 1980).

Surfactants such as sodium lauryl sulfate, which are a necessary ingredient in cosmetic formulations are very irritating to the skin and eyes. Proteins have been shown to exert a protective effect against irritation produced by various detergents (Teglia *et al.*, 1993). Generally, proteins with gelling, thickening and film-forming properties had a notable effect on reducing the irritancy of certain surfactants (Guillot

et al., 1983). The mechanism of this protective effect may simply be due to mechanical factors, or alternatively, adsorption on the outer surface of the skin. Proteins also protect against skin and eye irritation by their ability to bind certain surfactant molecules through ionic and hydrophobic interactions. Thus, proteins with a higher surface hydrophobicity show greater protection against these types of surfactants (Teglia *et al.*, 1993).

CHAPTER 1

Effects of Acetylation and Succinylation on the Physicochemical Properties of the Canola 12S Globulin

INTRODUCTION

The functional properties of a protein in a food or cosmetic system are related to its physicochemical characteristics, including factors such as molecular weight, amino acid composition and sequence, net charge and surface hydrophobicity. Particularly important in determining functionality are surface hydrophobicity, molecular weight and conformation, charge density, and molecular flexibility (Kim and Rhee, 1989). Therefore, in order to modify proteins with the objective of obtaining good functionality, knowledge of the physical character of a protein is imperative. In addition, certain physicochemical properties of a protein, determine a protein's ability to perform the functional properties unique to cosmetic systems. Specifically, the substantivity of a protein, or its ability to adhere to the skin or hair, is dependent upon molecular weight and net charge. Secondly, the anti-irritant potential of a cosmetic protein is influenced by its surface hydrophobicity.

Acylation through acetylation and succinylation causes an overall decrease in surface charge, plus dissociation of the spatial structure of a protein, followed by unfolding of polypeptide chains. Molecular flexibility is extremely important in determining the surface properties of proteins (Kim and Kinsella, 1986). Native globulins have limited molecular flexibility due to their relatively stable oligomeric structures (Schwenke *et al.*, 1986). Thus, dissociation of the native structure may improve the surface properties. Furthermore, modifications in net charge may be related to the protein solubility. The extensive alteration in charge brought about by

acylation has also been shown to be effective in reducing the levels of phytic acid (Thompson, 1987) and phenolics (Ponnampalam *et al.*, 1987) in canola protein isolates.

In this study, various physicochemical properties of the PMM and acylated concentrates were examined. Specifically, alterations in molecular weight, conformation, surface hydrophobicity and isoelectric points were analysed. In addition, changes in the amino acid profile were investigated. Finally, the levels of phytic acid, phenolics, and glucosinolates were also monitored, to aid in determining their suitability as a food or cosmetic ingredient.

MATERIALS AND METHODS

A. Canola Protein Isolation by the Protein Micellar Mass Procedure

Defatted canola meal (*Brassica napus*) was obtained from the Northern Light Canola Inc. (Sexsmith, AB). Samples of canola meal were stirred in 0.3 M NaCl (1:10 ratio) for 4 h then centrifuged for 10 min at 3000 g (4°C) using a Sorvall Refrigerated Centrifuge, Model RC-3 (DuPont Co., Wilmington, DE). The supernatant was collected and filtered through Whatman No. 4 filter paper under vacuum to remove any particulate matter. The supernatant was then concentrated using a 10^3 molecular weight cut-off Spiral Ultrafiltration cartridge in an Amicon UF unit (model RA 2000, Oakville, ON). The pressure was maintained at 20 psi with the volume of the supernatant being reduced eight times. The concentrated protein solution was diluted by six times its volume with refrigerated distilled water. The solution was then stored at 4°C for 16 h to allow the protein micelles to precipitate out of solution. The protein micelles were recovered by centrifugation at 3000 g for 30 min (4°C). The supernatant was discarded and the protein was collected and freeze-dried for 48 h to obtain the PMM (protein micellar mass).

B. Proximate Analysis

1. Moisture Content Determination

The moisture content was determined for canola meal and PMM using a vacuum oven as described in the AOAC Official Methods (AOAC, 1975).

2. Fat Content Determination

Fat content was determined for canola meal and PMM using a Soxhlet method according to the procedure outlined in the AOAC Official Methods (AOAC, 1975).

3. Total Ash Determination

Total ash content was determined for canola meal and PMM as described in the AOAC Official Methods (AOAC, 1975).

4. Protein Determination

The protein content of canola meal, PMM and all acylated proteins was determined by a micro-Kjeldahl method as described in the AOAC Official Methods (AOAC, 1975). The protein to nitrogen conversion factor used for canola was 5.8.

C. Preparation of Acylated Proteins

1. Succinylation

Succinylation was performed according to the method of Hoagland (1966). PMM (2% w/v), was dispersed in 0.075 M phosphate buffer pH 8. Succinic anhydride (Anachemia) was added in small increments with constant stirring over a 1 h period at levels of 2, 10, 50, and 100% of the weight of the PMM in the slurry. During the reaction, the pH was maintained between 8 and 8.5 with 3.5 M NaOH. Suspensions were then dialyzed using Spectra/Por Membrane Tubing (Spectrum,

Houston, TX) with a molecular weight cut-off of 12-14,000 for 24 h against distilled water at 4°C with 4 changes of water to remove the excess anhydride. A control was prepared in the same manner but the addition of succinic anhydride was omitted. Following dialysis the suspensions were freeze-dried to obtain the succinylated protein concentrates.

2. Acetylation

The method of Ponnampalam *et al.* (1990) was used with some modification for acetylation of the PMM. The PMM (2% w/v) was dispersed in distilled water and adjusted to pH 8.5 with 1 M NaOH. Acetic anhydride (Sigma) was added slowly with constant stirring (1, 2.5, 5 and 20% of the weight of the PMM in the slurry) for 1 h. The pH was maintained by the addition of 1 M NaOH. A control was prepared in the same manner without the addition of acetic anhydride. The suspension was dialyzed for 24 h using Spectra/Por Membrane Tubing (Spectrum, Houston, TX) with a molecular weight cut-off of 12-14,000 against distilled water at 4°C with 4 changes of water to remove excess reagent. The acetylated concentrate was recovered by lyophilization.

3. Determination of Extent of Modification

The degree of acylation was estimated by determination of free amino groups in the PMM as compared to the acylated proteins according to the method of Habeeb (1966) with modifications by Gueguen *et al.* (1990). A 1% (w/v) solution of PMM and acylated proteins was prepared in 0.05 M NaCl, pH 9.2, containing 0.29% SDS.

One mL of 0.05 M Na_2HPO_4 and 1 mL of 0.1% TNBS were added to 1 mL of protein solution. The solution was allowed to react at 60°C for 2 h; then 1 mL of 10% SDS was added followed by 0.5 mL of 1 M HCl. The absorbance of the solution was read at 335 nm against a blank prepared using identical conditions but omitting the protein.

D. Physicochemical Characteristics

1. Molecular Weight Determination by Gradient PAGE

The molecular weights of PMM and all acylated samples were estimated by nondissociating gradient PAGE according to the procedure of Robard *et al.* (1971). Bio-Rad Mini-PROTEAN II Gradient Ready Gels (Bio-Rad Laboratory, Hercules, CA) with an acrylamide concentration of 4-15% were used for all analyses. The stock solutions are outlined in Table 1.1 and the Sigma native protein molecular weight standards are described in Table 1.2.

Protein samples were prepared in the sample buffer at a concentration of 2.5 mg/mL; a sample (7.5 µL) was loaded into each slot and electrophoresed at 200 V constant voltage for 30 min. A Bio-Rad Mini-PROTEAN II electrophoresis apparatus (Richmond, CA) was used with a Bio-Rad Model 1000/500 Power Supply. The gels were then stained for 1 h on a shaking platform and destained for 2 h. Kodak Tech pan film (ESTAR-AH Base) was used to photograph the gels. The log molecular weights of the protein standards were plotted as a function of migration distance to establish a calibration curve (Appendix 1). The molecular weight of each of the protein bands was calculated using this calibration curve.

Table 1.1. Stock solutions for gradient PAGE

SOLUTION		SOLUTION COMPOSITION
0.5 M Tris-HCl, pH 6.8	0.6 g 6 mL	Tris deionized water adjusted to pH 6.8 with HCl, made up to 10 mL with deionized water and stored at 4°C
1% Bromophenol Blue	0.1 g 10 mL	bromophenol blue deionized water dissolve with gentle stirring
Sample buffer	5.8 mL 1.0 mL 0.8 mL 0.4 mL	deionized water 0.5 M Tris-HCl, pH 6.8 glycerol 0.5 % bromophenol blue
5x running buffer	15.0 g 72.0 g	Tris Glycine made up to 1 L with deionized water
Staining solution	400 mL 100 mL 1.0 g 500 mL	methanol acetic acid Coomassie blue R-250 deionized water filtered and stored at room temperature
Destaining solution	400 mL 100 mL 500 mL	methanol acetic acid deionized water

Table 1.2. Molecular weights of Sigma native protein standards

STANDARD	MOLECULAR WEIGHT (Dalton)
Urease	545,000 (hexamer) 272,000 (trimer)
Bovine serum albumin	132,000 (dimer) 66,000 (monomer)
Chicken egg albumin	45,000
α-lactalbumin	14,200

2. Determination of Isoelectric Point

The isoelectric point of each protein sample was determined according to the method of Winter and Anderson (1977). The PMM and modified proteins were solubilized in 0.025 M sodium citrate buffer at pH 8 and a concentration of 1 mg/mL. Isoelectric focusing was carried out on an LKB 2117 Multiphor apparatus (Sweden) with an LKB 2197 Constant Power Supply. The temperature was controlled at 10°C with a Haake circulating water bath (West Germany). An LKB Ampholine PAG (polyacrylamide gel) plate with a pH range of 3.5 to 9.5 was used. After the PAG plate was in position, an electrode strip soaked in 1 M NaOH was positioned at the cathode and a second electrode strip soaked in 1 M H_3PO_4 was placed at the anode. Ten µl of each of the protein samples and the Pharmacia standards with a pH range of 3.5-9.3 (Table 1.3) were applied on the surface of the gel.

Table 1.3. Isoelectric points of the Pharmacia protein standards used for isoelectric focusing.

STANDARD	pI (24°C)
Amyloglucosidase	3.5
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

The gel was then focused for 1.5 h at a constant power of 30 W but varying amperage (mA) and voltage (V) to allow the proteins to migrate to their isoelectric points. Upon completion, the electrode strips were immediately removed and the gel was fixed for 30 min, destained for 5 min, stained at 60°C in an air oven for 20 min, destained for 24 h and preserved for 1 h. The composition of all stock solutions used in isoelectric focusing is given in Table 1.4. The gel was dried at room temperature for 45 min and a Mylar plastic sheet was placed over the sticky gel surface. The gel was photographed using Kodak Tech pan film (ESTAR-AH Base).

Table 1.4. Solutions used in isoelectric focusing

SOLUTION		SOLUTION COMPOSITION
Fixing solution	57.5 g 17.25 g	trichloroacetic acid sulphosalicylic acid adjusted to 500 mL with distilled water
Destaining solution	500 mL 160 mL	ethanol acetic acid adjusted to 2000 mL with distilled water
Staining solution	0.46 g 400 mL	Coomassie Brilliant Blue R-250 destaining solution mixed and filtered through Whatman No. 1 filter paper
Preserving solution	300 mL 40 mL	destaining solution glycerol

A calibration curve (Appendix 2) was established by plotting the isoelectric points of the standards as a function of their migration distances from the cathode. The isoelectric points of the protein samples were determined by measuring the distance each band moved from the cathode.

3. Protein Conformational Changes

a. Spectrophotometry. The absorption and fluorescence spectra of the proteins were determined according to the procedure of Kim and Rhee (1989). The absorption spectra of 0.05% protein samples in 0.01 M phosphate buffer pH 8 were obtained using a Hewlett Packard 8451A Diode Array Spectrophotometer (Orangeville, ON) in the range of 240 to 350 nm in a 1 cm pathlength quartz cell. Fluorescence measurements were performed on a Perkin-Elmer LS-5 Fluorescence

Spectrophotometer (Coleman Instruments Division, Oak Brook, IL). Protein samples of 0.01% in 0.01 M phosphate buffer pH 8 were excited at 280 nm and the fluorescence emission spectra were recorded.

b. Differential Scanning Calorimetry (DSC) Analysis. The thermal properties of PMM and all acylated proteins were measured using a Dupont 990 Thermal Analyzer with a 910 Differential Scanning Calorimeter Cell Base (Westech Industrial Ltd., Missisauga, ON) in order to determine any conformational changes.

The DSC analysis procedure of Welsh (1988) was used with some modification. A slurry of canola protein in 0.1 M NaCl was prepared having 20% w/w total solids. Samples of the slurry weighing 10-15 mg were hermetically sealed into Dupont aluminum pans, coated on the interior with an inert polymer. The reference (a sealed empty pan) and the sample pan were placed in a high pressure DSC cell using a silicone heat-sink compound (Dow Corning Corp., Midland, MI) for better thermal conductivity. The protein sample was heated at a rate of 10°C/minute over a temperature range of 25°C to 120°C. Each sample was analyzed in triplicate.

The DSC Standard Analysis Data Program (Version 2.2C) was used to collect and analyze the thermal data. The results were expressed both in terms of the maximum heat flow into the protein (the thermal denaturation temperature, Td in °C) as well as the enthalpy of denaturation (the ΔH value expressed in Joules/g of sample). The thermal curve was plotted by a Hewlett Packard Model HP7470A graphics plotter.

c. Surface Hydrophobicity. The surface hydrophobicity of protein samples was determined using two fluorescent probes: 1-anilino-8-napthalenesulfonate (ANS, Sigma) to estimate the aromatic hydrophobicity; and cis-parinaric acid (CPA, Calbiochem) to estimate the aliphatic hydrophobicity. A solution of ANS was prepared according to the method of Hayakawa and Nakai (1985); 8 mM magnesium ANS was solubilized in 0.02 M phosphate buffer, pH 7.4. A solution of CPA was prepared according to the procedure of Kato and Nakai (1980), which consisted of equimolar (3.6 mM) CPA and butylated hydroxytoluene in ethanol.

Protein samples were serially diluted with 0.01 M phosphate buffer pH 8.0 to obtain a range of protein concentrations from 0.015 to 0.60 mg/mL. A 10 µL aliquot of ANS or CPA was added to 2 mL of each sample, and the relative fluorescence intensity was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer (Coleman Instruments Division, Oak Brook, IL) using a slit width of 0.5 nm and a fixed scale of 1.0. Temperature was maintained at 20°C with a Haake-G water bath. Wavelengths of excitation and emission were 390 and 470 nm for ANS plus 325 and 420 nm for CPA. All samples were analyzed in triplicate. The fluorescence intensity of each solution without the probe was subtracted from that with the probe to obtain the net fluorescence intensity (FI) at each protein concentration. The initial slope of a plot of fluorescence intensity as a function of protein concentration was used as an index of protein surface hydrophobicity (So).

4. Amino Acid Analysis

a. High Performance Liquid Chromatography. The amino acid profile of the PMM and acylated proteins was determined according to the procedure of Andrews and Baldar (1985). A PMM sample (50-300 mg) was placed into the bottom of a 100 mL round-bottom flask. Hydrolysis was carried out in 50 mL of 6 N HCl containing 50 mg phenol under reflux at approximately 110°C for 23 hours. Following hydrolysis the condenser was rinsed with 0.2 M sodium citrate buffer, pH 2.2, and the sample was allowed to cool. The pH of the hydrolysate was brought to 2.2 using 7.5 M NaOH with the temperature maintained below 40°C in an ice bath. The solution was made up to 200 mL with sodium citrate buffer, pH 2.2, and 2 mL of this solution was filtered through a 0.22 µm centrifugal microfilter before loading onto the amino acid analyzer.

All analyses were performed using a LKB 4151 Alpha Plus Amino Acid Analyzer (LKB Biochrom Ltd., Cambridge, UK) equipped with a 200 x 4.6 mm stainless-steel column packed with Ultrapac 8 (8 µm ± 0.5 µm) cation-exchange resin. Detection was performed at two wavelengths: 570 nm for amino acids and 440 nm for imino acids. The signal from both detection wavelengths was summed before proceeding to an LKB Recording Integrator.

b. Tryptophan Determination. To account for the loss of tryptophan during acid hydrolysis, tryptophan levels in the proteins were determined separately according to the spectrophotometric method of Messineo and Musarra (1972).

E. Antinutritional Factors

1. Phytic Acid Determination

Analysis of phytic acid content of PMM and acylated proteins was carried out according to the method outlined by Latta and Eskin (1980).

2. Determination of Phenolic Compounds

Analysis of the phenolic compounds in the PMM and acylated proteins was performed by a modification of the method of Dorrell (1976). Lowry reagent was substituted for Folin-Denis reagent according to a modification by Schanderl (1970) of the method of Swain and Hillis (1959).

Samples (50 mg) were refluxed in 12.5 mL of ethanol at pH 4 for 30 min, allowed to cool, then centrifuged on a Sorvall GLC-1 benchtop centrifuge (Dupont Co., Wilmington, DE) for 5 min at 2000 g. The supernatant was then adjusted to a volume of 25 mL with distilled water. A 0.5 mL aliquot of the supernatant was diluted to 7 mL with distilled water in a graduated test tube. Lowry reagent (0.5 mL) was added to this solution and the tube was allowed to sit for 3 min. One mL of saturated sodium carbonate solution was added. The entire solution was subsequently made up to a 10 mL volume with distilled water and allowed to sit for 1 h at room temperature. The absorbances of the samples and the tannic acid standards were read at 750 nm on a Hewlett Packard 8451A Diode Array Spectrophotometer (Orangeville, ON) against a blank of distilled water.

3. Analysis of Glucosinolates

Glucosinolate analysis of the PMM and protein samples was performed by gas-liquid chromatography according to the method of Slominski and Campbell (1987).

D. Statistical Analysis

The statistical analyses were performed using a SAS statistical analysis software program package. Significant differences among treatments were determined by Duncan's Multiple Range Test ($p \leq 0.05$).

RESULTS AND DISCUSSION

A. Proximate Analysis

Proximate analyses were performed on the canola meal and the PMM to monitor the effects of the PMM procedure on the moisture, fat, ash, and protein contents. The results of the proximate analyses are presented in Table 1.5. Canola meal was found to contain 34.43% protein, which is in the range of the findings of Han (1994) and Burgess (1991) who reported 32.5% and 35.6% respectively. Literature values of the ash content of canola meal ranged from 6.3% (Bell, 1989) to 9.94% (Burgess, 1991). The experimental value of 6.65% in this study is comparable to these values. The fat content of the meal was 3.24%, which was slightly lower than obtained by Burgess (1991) and Han (1994) (4.2% and 4.4% respectively).

The PMM contained 3.26% moisture, 15.21% fat, 1.90% ash and 77.56% protein. The PMM procedure reduced the moisture and ash contents, while concentrating the protein and fat.

TABLE 1.5. Proximate analysis of canola meal and PMM.

COMPONENT	CANOLA MEAL[1,2]	PMM[1,2]
Moisture	7.69 ± 0.12	3.26 ± 0.21
Fat	3.24 ± 0.08	15.21 ± 0.70
Ash	6.65 ± 0.28	1.90 ± 0.04
Protein	34.43 ± 3.11	77.56 ± 0.92

[1] Values expressed on a % dry weight basis.
[2] Each value represents a mean of two determinations.

B. Extent of Acylation

Succinic anhydride added to the PMM at levels of 2, 10, 50 and 100% of the weight of the protein acylated 3, 48, 53 and 61% respectively of the ε-amino groups (Figure 1.1). These samples were then referred to as 3% S-PMM, 48% S-PMM, 53% S-PMM, and 61% S-PMM. Acetic anhydride, added at levels of 1, 2.5, 5 and 20% of the protein in the slurry, modified 16, 26, 42 and 62% of the ε-amino groups (Figure 1.2). These samples were then referred to as 16% A-PMM, 26% A-PMM, 42% A-PMM and 62% A-PMM. These results are slightly lower than some reported by other researchers. For instance, with a 100% w/w level of addition as was used in this study, greater than 80% succinylation was achieved by Canella *et al.* (1979), with sunflower proteins, and Franzen and Kinsella (1976a) with soy proteins. However, the lower levels of acylation may be due to the poor solubility of PMM, or perhaps its hydrophobic character (Barber and Wartheson, 1982).

Acetic anhydride was found to be a more effective acylating reagent than succinic anhydride, which is in agreement with other studies (Ball and Winn, 1982; Ponnampalam *et al.*, 1990). As acetic anhydride is a liquid, it is mixed more readily into the reaction medium. To achieve the same level of succinylation as that of acetylation, it is necessary to employ more succinic anhydride and increase the reaction time to facilitate dissolution of the anhydride (Ponnampalam *et al.*, 1990).

FIGURE 1.1. Extent of succinylation as a function of ratio of succinic anhydride to protein.

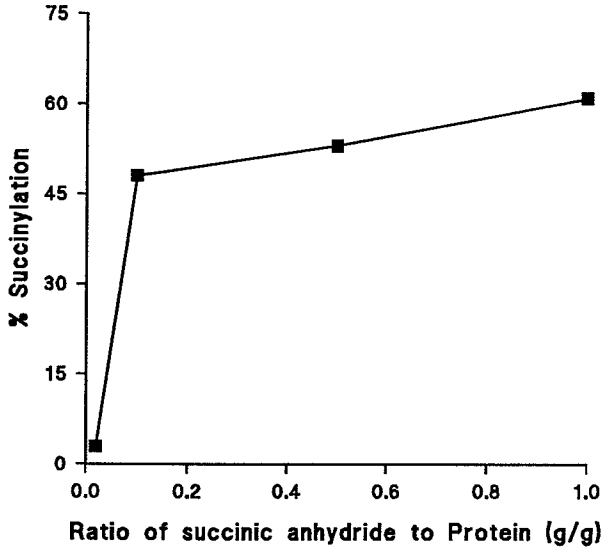
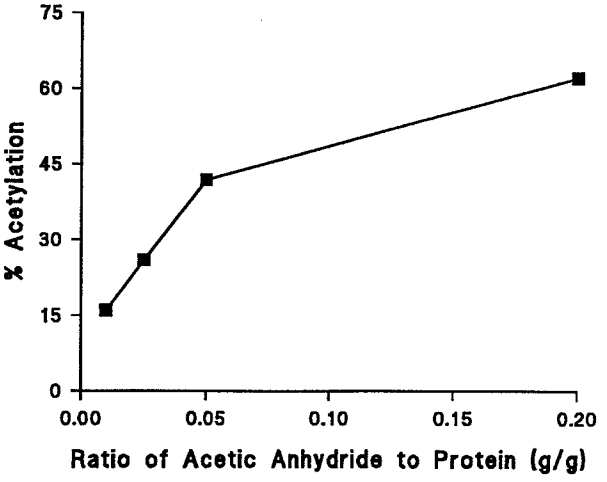


FIGURE 1.2. Extent of acylation as a function of ratio of acetic anhydride to protein.



C. Physicochemical Characteristics

1. Molecular Weight Determination

The molecular weights of the PMM and all modified proteins are given in Table 1.6 and the electrophoretic patterns are presented in Figure 1.3. The PMM had an intense band at 500,000 and a lighter band at 850,000. These probably represent the 15-17S components which are aggregates of the 12S globulin. As the level of succinylation increased, the molecular weight of the bands decreased. This corroborates the findings of Kabirullah and Wills (1982), who reported that the major band of native sunflower proteins moved further into the gel with increasing degrees of acylation. This effect is likely due to dissociation of the aggregates as a result of acylation. At a level of 53% succinylation, the 12S band was still evident, but a band at 50,000, which corresponds to the 2-3S subunit, also appeared. At the 62% level of succinylation, the 12S band disappeared and only the 2-3S band was present, indicating complete dissociation of the protein into subunits. This may be expected, as Schwenke *et al.* (1986) had previously characterized 60% modification to be the critical level for globulins from plant seeds. Schwenke *et al.* (1991) reported similar results with fababean protein, in which the 11S band showed increased mobility up to 60% modification. At this point the appearance of bands which moved more quickly indicated dissociation of the oligomeric protein components.

The acetylated proteins exhibited a similar banding pattern, but the extent of dissociation was not as pronounced, as indicated by a lesser decrease in molecular weight as compared with succinylation. Acetylation does not cause as extensive unfolding and dissociation as succinylation due to decreased charge effects. The 7S

fraction was not apparent on the gel, which may be expected as Prakash and Rao (1986) have determined the 7S component to be transient in *Brassica* spp.

TABLE 1.6. Molecular weights of PMM and all acylated proteins estimated by gradient PAGE.

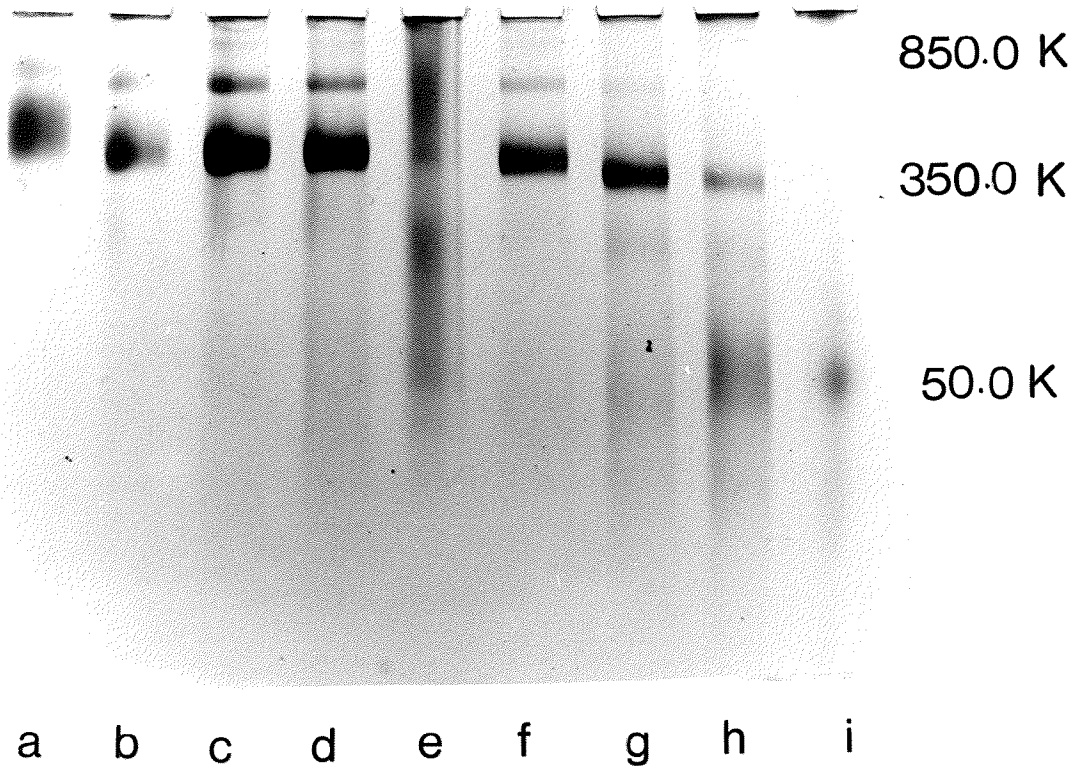
SAMPLE	MOLECULAR WEIGHT[1] (Daltons)
PMM	850,000 500,000
3% succinylation	750,000 400,000
48% succinylation	750,000 350,000
53% succinylation	350,000 50,000
61% succinylation	50,000
16% acetylation	750,000 400,000
26% acetylation	750,000 400,000
42% acetylation	750,000 400,000
62% acetylation	-

[1] Each value represents a mean of two determinations.

The stepwise dissociation pattern resulting from acylation observed in this study has been frequently cited by other researchers, including Narayana and Rao (1991) with winged bean protein, Venktesh and Prakash (1994) with sunflower seed protein, and Gueguen *et al.* (1990) with rapeseed protein.

FIGURE 1.3. Gradient PAGE electrophoregram of PMM and all acylated proteins.

Lane	a.	PMM
	b.	16% A-PMM
	c.	26% A-PMM
	d.	42% A-PMM
	e.	62% A-PMM
	f.	3% S-PMM
	g.	48% S-PMM
	h.	53% S-PMM
	i.	61% S-PMM


850.0 K
350.0 K
50.0 K
a
b
c
d
e
f
g
h
i

2. Isoelectric Point Determination

The isoelectric focusing patterns of the PMM and modified proteins are shown in Figure 1.4 and the estimated isoelectric points are presented in Table 1.7.

A single band was observed for PMM, which corresponds to an isoelectric point of 7.0. This is in agreement with the value of 7.2 reported in the literature for the 12S canola globulin (Schwenke *et al.*, 1983).

The succinylated proteins exhibited a successive decrease in the range of isoelectric points as the extent of acylation increased. The band with the highest intensity was found at pH 4.9 for the first three levels of succinylation. This increase in net negative charge is a result of the replacement of the positively charged amino groups with the carboxyl anions of the succinate half-amide (Canella *et al.*, 1979). At the highest level of succinylation (61% S-PMM), the most intense band corresponded to an isoelectric point of 5.2, which is slightly higher than the previous level of succinylation. Gueguen *et al.* (1990) reported a similar increase in isoelectric point with excessive succinylation, and the band was assumed to correspond to the modified subunits.

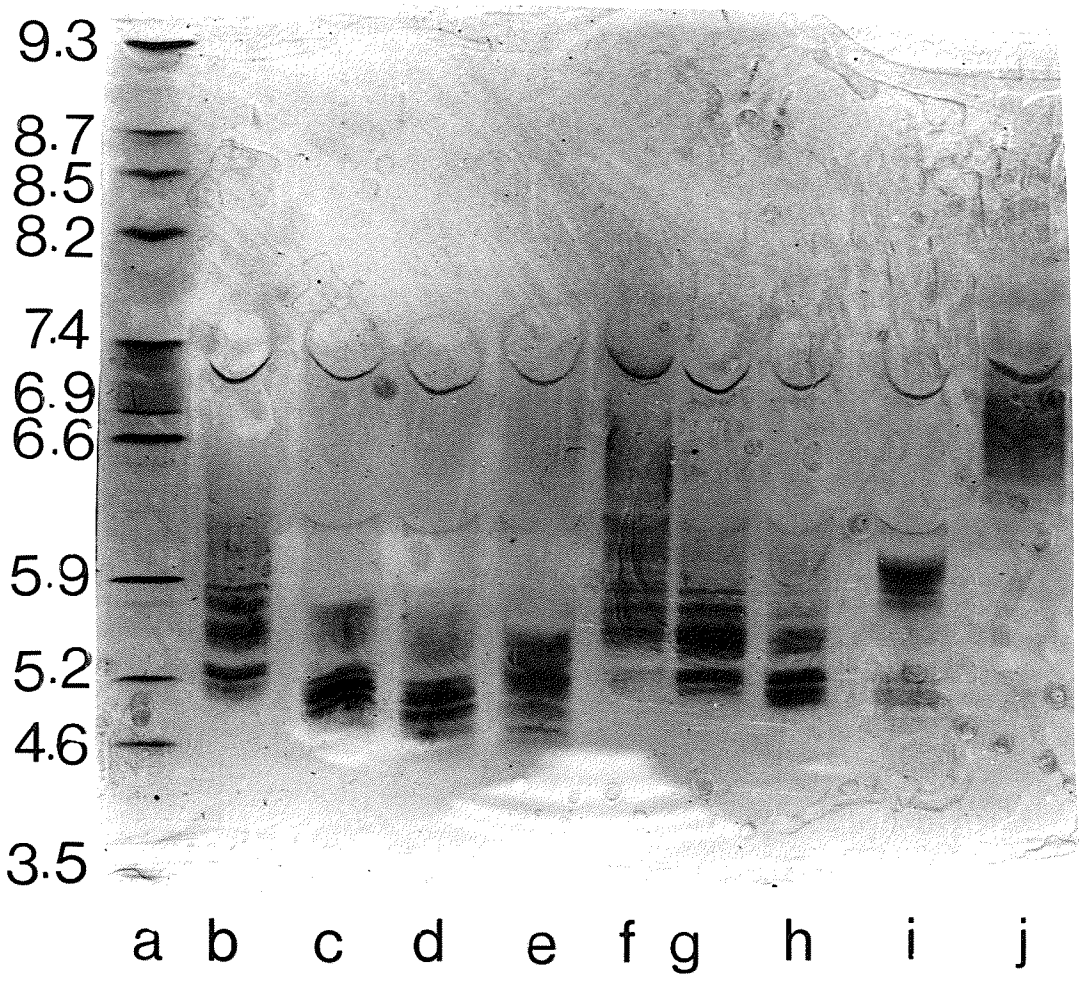
A similar pattern of decreasing isoelectric point with increasing level of acylation was noticed with the acetylated proteins. However, the decrease in isoelectric point was not as pronounced as those observed with succinylation, due to the acetyl group being neutral rather than anionic.

TABLE 1.7. Isoelectric points for PMM and all acylated proteins determined by isoelectric focusing.

SAMPLE	ISOELECTRIC POINT (pH)
PMM	7.1
3% succinylation	5.5
	5.3
	4.9
48% succinylation	5.5
	4.9
	4.6
53% succinylation	4.9
	4.6
61% succinylation	5.2
16% acetylation	6.1
	5.5
26% acetylation	5.7
	5.4
	5.1
42% acetylation	5.3
	5.1
	5.0
62% acetylation	5.8
	4.9

FIGURE 1.4. Isoelectric focusing patterns for standards, PMM plus all acylated proteins.

Lane		
	a.	standards
	b.	3% S-PMM
	c.	48% S-PMM
	d.	53% S-PMM
	e.	61% S-PMM
	f.	16% A-PMM
	g.	26% A-PMM
	h.	42% A-PMM
	i.	62% A-PMM
	j.	PMM


pH
9.3
8.7
8.5
8.2
7.4
6.9
6.6
5.9
5.2
4.6
3.5
a b c d e f g h i j

3. Protein Conformational Changes

a. Spectrophotometry. The second derivatives of the UV spectra of PMM and succinylated proteins are given in Figure 1.5, and that of the PMM and acetylated proteins are shown in Figure 1.6.

In both acetylated and succinylated proteins there was a progressive shift in λmax towards shorter wavelengths (blue shift) with increasing degrees of modification. These results are consistent with those of Kim and Kinsella (1986), plus Kim and Rhee (1989). According to Freifelder (1982), this blue shift indicates that the aromatic chromophores of the protein were shifted to a more polar environment as a result of dissociation and unfolding.

The λmax of the fluorescence emission spectra of PMM and all modified proteins is given in Table 1.8. The λmax of the fluorescence emission of PMM was determined to be 340 nm. As the degree of modification increased, a progressive increase in the λmax (red shift) was observed. The maximum wavelength shift was an increase to 347 nm for the 61% S-PMM, and 346 nm for the 62% A-PMM. This indicates that the tryptophan residues in the protein became exposed to a more polar environment because of dissociation of the native oligomeric structure (Kim and Kinsella, 1986). Lakkis and Villota (1992) observed similar shifts in fluorescence emission after acetylation of casein, BSA and whey protein.

FIGURE 1.5. Second derivative of UV/Vis spectrum of PMM and succinylated proteins.

a. PMM
b. 3% S-PMM
c. 48% S-PMM
d. 53% S-PMM
e. 61% S-PMM

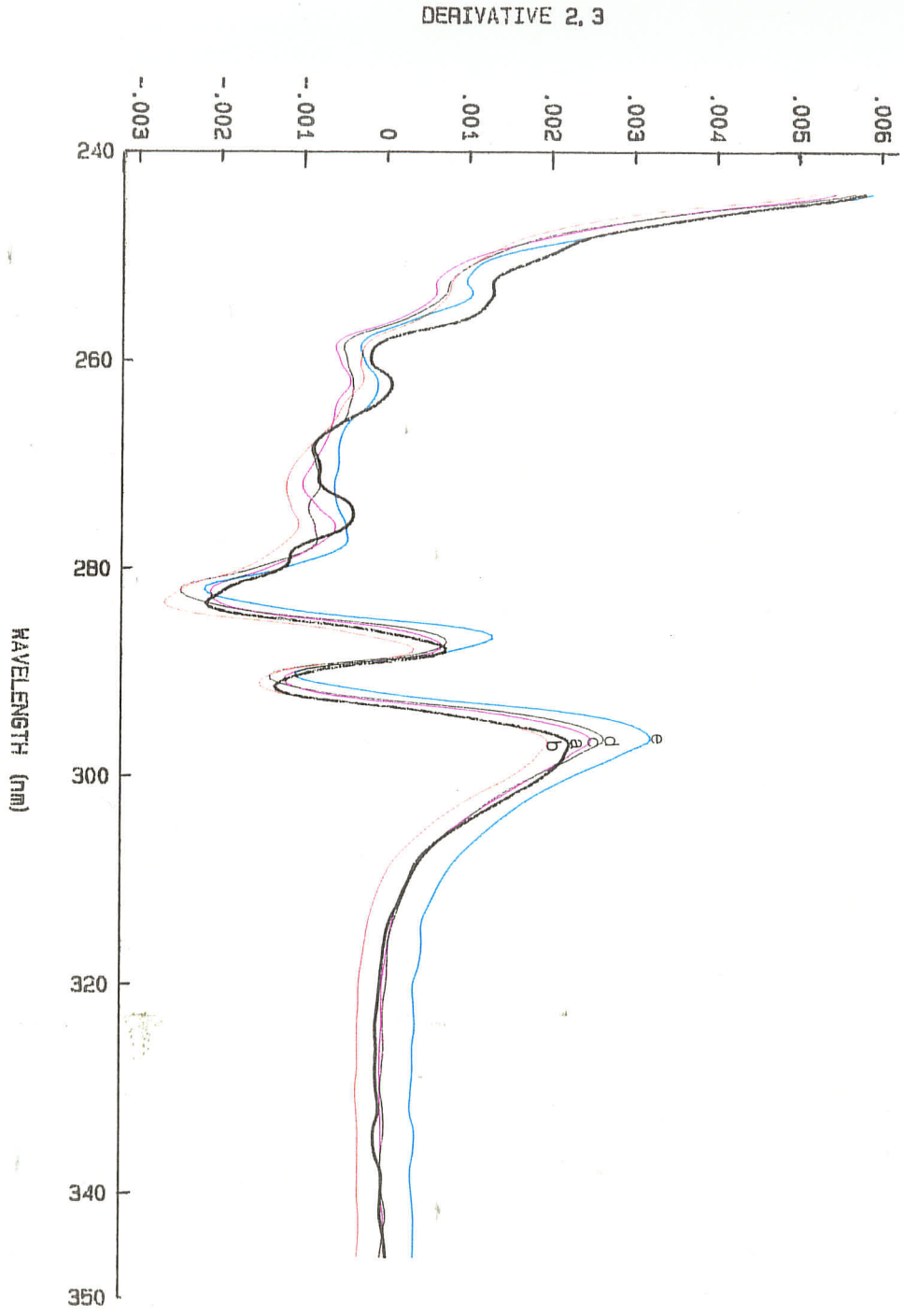

DERIVATIVE 2, 3
-.003
-.002
-.001
0
.001
.002
.003
.004
.005
.006
240
260
280
300
320
340
350
WAVELENGTH (nm)

FIGURE 1.6. Second derivative of UV/Vis spectrum of PMM and acetylated proteins.

a. PMM
b. 16% A-PMM
c. 26% A-PMM
d. 42% A-PMM
e. 62% A-PMM

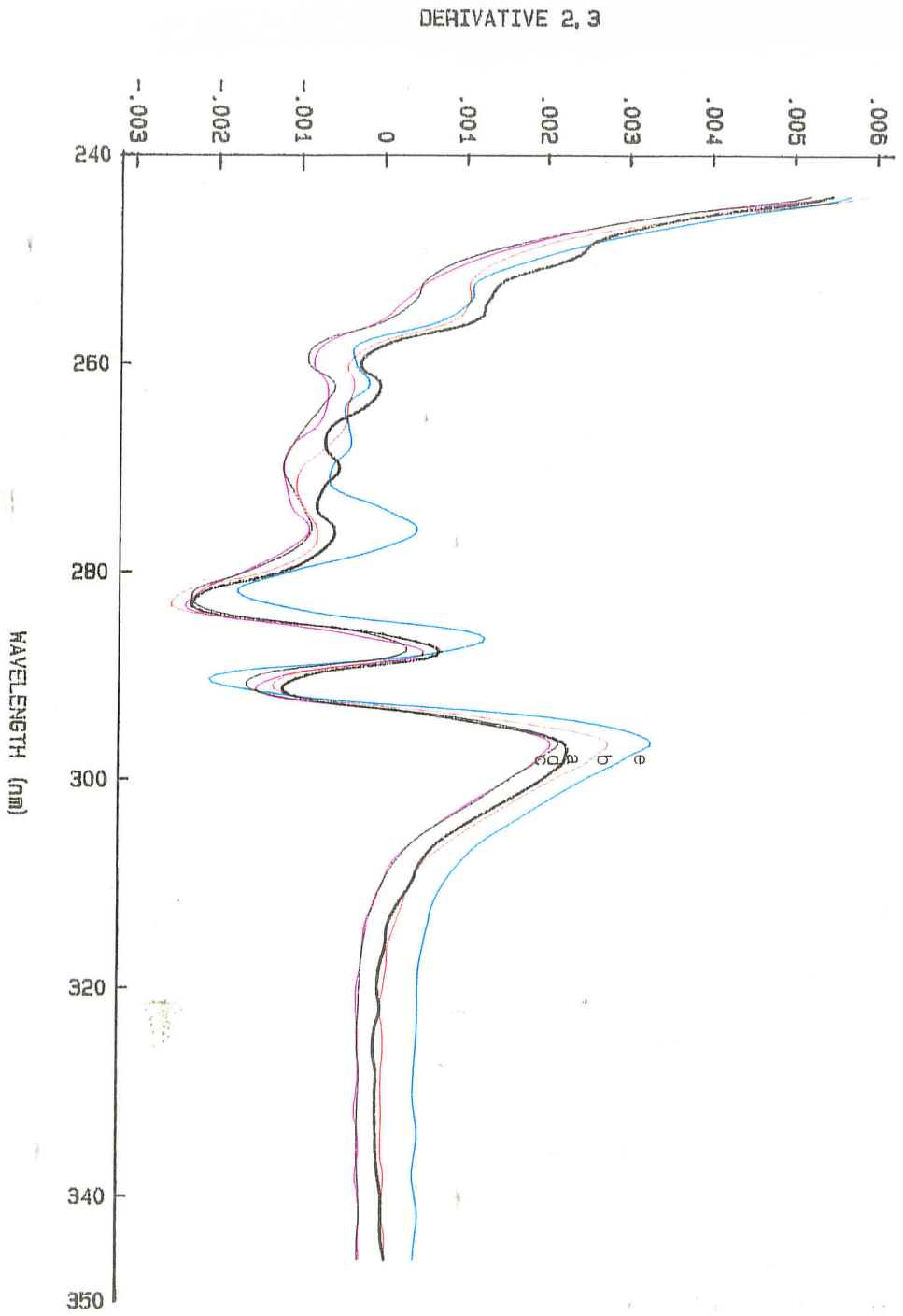

DERIVATIVE 2, 3
-.003
-.002
-.001
0
.001
.002
.003
.004
.005
.006
240
260
280
300
320
340
350
WAVELENGTH (nm)
a
b
c
d
e

TABLE 1.8. Maximum λ of fluorescence emission spectra of PMM and all acylated proteins.

SAMPLE	λMAX OF FLUORESCENCE EMISSION[1]
PMM	340 ± 1
3% succinylation	340 ± 1
48% succinylation	341 ± 1
53% succinylation	344 ± 1
61% succinylation	348 ± 1
16% acetylation	340 ± 1
26% acetylation	340 ± 0
42% acetylation	341 ± 1
62% acetylation	346 ± 0

[1] Each value represents a mean of two determinations.

b. Differential Scanning Calorimetry (DSC) Analysis. The thermal parameters of a protein as determined by DSC are useful indices of the degree of conformational changes of proteins (Arntfield and Murray, 1981). The thermal denaturation temperature (Td) and the enthalpy of denaturation (ΔH) are the two most important parameters in DSC analysis. The values for the Td and ΔH of the PMM and modified proteins are presented in Table 1.9. The analysis of variance of the thermal denaturation temperature is shown in Appendix 3a and the analysis of variance of the enthalpy of denaturation is shown in Appendix 3b.

TABLE 1.9. The enthalpy of denaturation (ΔH in Joules/g) and the thermal denaturation temperature (Td in °C) for PMM and all modified proteins[1].

SAMPLE	Td (°C)[2]	ΔH (Joules/g)[2]
PMM	87.56 ± 0.16[b]	10.97 ± 0.52[a]
3% succinylation	87.14 ± 0.32[c]	9.78 ± 0.78[b]
48% succinylation	84.24 ± 0.37[d]	2.41 ± 0.61[e]
53% succinylation	83.58 ± 0.22[e]	1.06 ± 0.13[f]
61% succinylation	-	0
16% acetylation	89.03 ± 0.16[a]	8.01 ± 0.39[c]
26% acetylation	89.00 ± 0.23[a]	8.45 ± 0.41[c]
42% acetylation	88.79 ± 0.23[a]	5.50 ± 0.49[d]
62% acetylation	-	0

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents a mean of three determinations.

The ΔH is a measurement of the enthalpy required for thermal denaturation of a protein. In this study, PMM was found to have a ΔH of 10.97 Joules/g. This value is in agreement with the ΔH of the PMM of Han (1994).

The ΔH values decreased significantly at each level of succinylation. At the highest level of succinylation the endotherm completely disappeared, indicating complete denaturation. For the acetylated proteins, a significant decrease in ΔH values was also observed, but this effect was not as dramatic as those of the succinylated proteins. At the highest level of acetylation, the endotherm also

disappeared. This supports the theory of Schwenke *et al.* (1991) that 60% modification is the critical level for denaturation of plant proteins.

The temperature of denaturation (Td) denotes the temperature required to denature a protein. The PMM had a Td of 87.6°C, and this value decreased significantly with each increasing level of succinylation, indicating destabilization of the protein structure. The first three levels of acetylation exhibited a significant increase in Td from the PMM.

c. Surface Hydrophobicity. The surface hydrophobicity of a protein is an index of the number of hydrophobic groups on the surface of a protein in contact with the polar aqueous environment. Surface properties of a protein are significant, as they are indicative of its capacity for intermolecular interaction, thereby influencing its functionality. According to Hayakawa and Nakai (1985), protein hydrophobicity may be classified into two types, aromatic and aliphatic, depending on the presence of aromatic and aliphatic side chain residues. Several researchers have correlated surface hydrophobicity with functional properties. Li-Chan *et al.* (1985) reported that both aromatic and aliphatic hydrophobicity were significant predictors of emulsifying and fat-binding properties of salt soluble muscle proteins. However, a study by Hayakawa and Nakai (1985) on the thermal properties of ovalbumin found no difference between the two types of hydrophobicity measurements. Further research by Nakai and Li-Chan (1988) indicated that aromatic hydrophobicity showed a significant correlation with protein insolubility.

The results for aromatic and aliphatic hydrophobicities are given in Table 1.10. The analysis of variance of the aromatic and aliphatic surface hydrophobicity is shown in Appendix 4a and Appendix 4b respectively. The aromatic hydrophobicity of PMM was found to be 97.5. As the level of succinylation increased, the aromatic hydrophobicity showed a significant decrease, to a value of 58.1 at the highest level of modification. This corroborates the research of Paulson and Tung (1987) on canola proteins. In their study a decrease in aromatic hydrophobicity of canola protein isolates was noticed with an increase of succinylation.

TABLE 1.10. The aromatic and aliphatic surface hydrophobicity of PMM and all acylated proteins[1].

SAMPLE	AROMATIC HYDROPHOBICITY[2]	ALIPHATIC HYDROPHOBICITY[2]
PMM	97.51 ± 1.79^{bc}	222.39 ± 16.11^{cd}
3% succinylation	85.12 ± 1.72^{d}	170.20 ± 8.19^{e}
48% succinylation	55.52 ± 3.42^{f}	214.23 ± 17.36^{cd}
53% succinylation	62.31 ± 1.52^{e}	238.33 ± 18.14^{c}
61% succinylation	58.11 ± 0.82^{ef}	283.89 ± 6.35^{b}
16% acetylation	92.97 ± 0.49^{c}	205.50 ± 7.59^{d}
26% acetylation	84.44 ± 0.92^{d}	175.10 ± 2.27^{e}
42% acetylation	101.12 ± 4.91^{b}	207.24 ± 6.99^{d}
62% acetylation	322.57 ± 2.94^{a}	417.05 ± 19.66^{a}

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents a mean of three determinations.

Paulson and Tung (1987) suggested that as there is a higher frequency of charged groups resulting from succinylation which are assumed to be on the surface of the protein, it is likely to result in a lower frequency of nonpolar groups on the surface. This is supported by their report that with increasing levels of succinylation charge frequency and electronegativity increased while the surface hydrophobicity decreased. For the acetylated proteins, the aromatic hydrophobicity initially decreased before exhibiting a significant increase with the 62% A-PMM. Kim and Rhee (1989) observed a similar pattern with the aromatic hydrophobicity of acetylated soy proteins. This increase at high levels of acetylation could be due to dissociation of the subunits as positive charges are replaced with neutral charges.

This resulted in a repulsion between subunits which would have made previously buried hydrophobic groups accessible to binding by ANS.

The aliphatic hydrophobicity of PMM was found to be 222.4. The aliphatic hydrophobicity of the succinylated proteins initially decreased, then exhibited a significant increase at the highest level of modification. This is in accordance with the results of Ma *et al.* (1986) on the surface hydrophobicity of wheat gluten. In this study, the aliphatic hydrophobicity of the acetylated proteins demonstrated a similar pattern as with their aromatic hydrophobicity, by decreasing initially then increasing.

D. Amino Acid Analysis

The results of the amino acid analysis of PMM and modified proteins are given in Table 1.11 and 1.12 and the results of the tryptophan determination are shown in Table 1.13.

The amino acid profile appeared to remain unchanged with the exception of a slight decrease in lysine following succinylation and acetylation of the proteins and a decrease in proline following succinylation. Several other researchers have investigated the effects of acylation on the amino acid content of proteins. In addition, Franzen and Kinsella (1976a) and Kabirullah and Wills (1982) reported little change in amino acid profile following acylation of soy proteins and sunflower proteins respectively. However, Franzen and Kinsella (1976b) found that there was no apparent destruction of amino acids following acylation of leaf proteins. On the other hand, Ma and Wood (1987) noticed a slight decrease in phenylalanine in

succinylated oat proteins, and a decrease in valine, leucine, isoleucine, phenylalanine and cystine in acetylated oat proteins.

Franzen and Kinsella (1976a) suggested that the effect of acylation on the nutritional value of proteins may be overcome by reducing the extent of acylation, as well as by supplementing the diet with lysine. Furthermore, acylated proteins which are intended for use as functional ingredients do not provide a significant source of nutritive protein in the foods into which they are incorporated (Franzen and Kinsella, 1976a).

TABLE 1.11. Amino acid analysis of PMM and succinylated proteins[1].

AMINO ACID	SAMPLE				
	PMM	3% S-PMM	48% S-PMM	53% S-PMM	61% S-PMM
aspartic acid	8.48	9.13	9.16	9.26	8.64
threonine	3.43	3.69	3.48	3.48	3.53
serine	5.04	5.42	5.29	5.35	5.20
glutamic acid	18.99	18.05	20.10	20.21	19.98
proline	8.29	8.62	8.13	8.29	7.83
glycine	6.01	6.07	6.34	6.29	6.21
alanine	4.24	4.64	4.52	4.50	4.41
cystine	1.78	1.51	1.49	1.49	1.74
valine	3.87	3.96	4.09	4.09	4.76
methionine	1.76	2.33	2.26	2.20	1.64
isoleucine	3.42	3.24	2.96	2.88	3.59
leucine	8.18	8.05	8.10	8.03	8.20
tyrosine	2.91	3.07	2.97	3.01	2.81
phenylalanine	5.22	5.33	5.15	5.15	5.10
histidine	3.27	2.59	2.58	2.59	2.65
lysine	4.66	3.77	2.70	2.47	2.94
arginine	8.31	8.33	8.31	8.37	8.44

[1] Expressed as percentage of total amino acids in proteins.

TABLE 1.12. Amino acid analysis of PMM and acetylated proteins[1].

AMINO ACID	PMM	SAMPLE 16% A-PMM	26% A-PMM	42% A-PMM	62% A-PMM
aspartic acid	8.48	9.81	9.81	9.71	9.47
threonine	3.43	3.83	3.83	3.76	3.70
serine	5.04	5.61	5.61	5.54	5.56
glutamic acid	18.99	18.84	18.84	18.91	18.62
proline	8.29	6.28	6.28	6.56	7.63
glycine	6.01	6.07	6.07	6.07	6.21
alanine	4.24	4.87	4.87	4.95	4.79
cystine	1.78	1.19	1.19	1.27	1.39
valine	3.87	4.30	4.30	4.18	3.75
methionine	1.76	2.25	2.25	2.27	2.27
isoleucine	3.42	3.72	3.72	3.65	3.19
leucine	8.18	8.10	8.10	8.12	8.14
tyrosine	2.91	3.00	3.00	2.97	3.34
phenylalanine	5.22	5.21	5.21	5.16	5.18
histidine	3.27	2.71	2.71	2.74	2.31
lysine	4.66	3.92	3.92	3.92	4.06
arginine	8.31	7.74	7.74	7.75	8.04

[1] Expressed as percentage of total amino acids in proteins.

TABLE 1.13. Tryptophan content of PMM and all acylated proteins.

SAMPLE	TRYPTOPHAN CONTENT[1,2]
PMM	0.020 ± 0.001
3% succinylation	0.021 ± 0.000
48% succinylation	0.023 ± 0.001
53% succinylation	0.024 ± 0.001
61% succinylation	0.025 ± 0.001
16% acetylation	0.020 ± 0.001
26% acetylation	0.020 ± 0.001
42% acetylation	0.021 ± 0.000
62% acetylation	0.024 ± 0.001

[1] Values expressed as µg tryptophan/µg protein
[2] Each value represents a mean of three determinations.

E. Antinutritional Factors

1. Phytic Acid

The phytic acid contents of PMM and all acylated proteins are presented in Table 1.14. The analysis of variance of the phytic acid content is shown in Appendix 5. The PMM was found to have a phytic acid content of 1.54%, which is similar to the results of Welsh (1988) who reported a range of 0.96 to 1.29% phytic acid in PMM.

Both succinylation and acetylation caused a steady decrease in the phytic acid content of the proteins. This effect was significant at the 48, 53, and 61% succinylations, but only at the 62% level of acetylation. These findings corroborate

the research of Thompson (1987) on rapeseed protein isolates. Thompson (1987) suggested that at a high level of modification, extensive changes in protein conformation due to an increase in net negative charges can cause steric hindrance to the binding of phytic acid to the protein. In addition, at the high levels of modification, the ternary phytic acid-cation-protein complex may have been destabilized. This effect would be due to the excessive amount of alkali added to the reaction mixture in order to stabilize the pH (Thompson, 1987).

TABLE 1.14. Phytic acid content of PMM and all acylated proteins[1].

SAMPLE	PHYTIC ACID CONTENT (%)[2]
PMM	1.54 ± 0.13^{a}
3% succinylation	1.36 ± 0.04^{ab}
48% succinylation	1.02 ± 0.11^{c}
53% succinylation	1.00 ± 0.11^{c}
61% succinylation	0.60 ± 0.14^{d}
16% acetylation	1.39 ± 0.09^{ab}
26% acetylation	1.59 ± 0.01^{a}
42% acetylation	1.39 ± 0.06^{ab}
62% acetylation	0.27 ± 0.12^{b}

1 Values followed by the same letter are not significantly different ($p \leq 0.05$).
2 Each value represents a mean of two determinations.

2. Phenolic Compounds

The phenolic contents of PMM and all acylated proteins are given in Table 1.15. The analysis of variance of phenolic content is shown in Appendix 6. The phenolic content of PMM was found to be 0.82%, which is higher than the range of 0.005 to 0.019% reported by Welsh (1988).

At the 3% S-PMM the phenolic levels exhibited a significant decrease. However, at the two highest levels of modification, the phenolics increased significantly. This was attributed to dissociation products of the protein due to extensive succinylation being extracted during the ethanolic extraction step of the phenolics' determination procedure. These dissociation products may then bind to the Folin reagent to give a false positive reading.

Acetylation caused an overall significant decrease in the levels of phenolics. This is in agreement with the findings of Ponnampalam *et al.* (1987). Ponnampalam *et al.* (1987) hypothesized that an increase in net negative charge as a result of acetylation and the introduction of bulky acyl groups could affect the degree of protein-phenol interactions. In this study, if protein-phenol interactions were inhibited, this would allow for their removal during the dialysis step following acetylation.

TABLE 1.15. Phenolic content of PMM and all acylated proteins[1].

SAMPLE	PHENOLIC CONTENT (%)[2]
PMM	0.82 ± 0.03^{c}
3% succinylation	0.38 ± 0.09^{d}
48% succinylation	0.78 ± 0.04^{c}
53% succinylation	1.16 ± 0.09^{b}
61% succinylation	1.73 ± 0.03^{a}
16% acetylation	0.37 ± 0.02^{d}
26% acetylation	0.44 ± 0.01^{d}
42% acetylation	0.35 ± 0.02^{d}
62% acetylation	0.42 ± 0.01^{d}

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents a mean of three determinations.

3. Glucosinolates

The glucosinolate contents of PMM and all acylated proteins are given in Tables 1.16 and 1.17.

Both acetylation and succinylation caused an overall decrease in glucosinolates to nondetectable levels in all but the 16% A-PMM, 26% A-PMM and 62% A-PMM. Thompson and Cho (1984) also reported glucosinolates at non-detectable levels in acetylated rapeseed protein isolates. Glucosinolates are readily soluble in the aqueous environment used for acetylation and succinylation. Therefore, they are easily removed during dialysis.

TABLE 1.16. Glucosinolate content of PMM and succinylated proteins.

INTACT GLUCOSINOLATES	PMM[1]	SAMPLE 3% S-PMM	48% S-PMM	53% S-PMM	61% S-PMM
allyl	ND[2]	ND	ND	ND	ND
-butenyl	0.55	ND	ND	ND	ND
-pentenyl	0.30	ND	ND	ND	ND
-OH-3-butenyl	0.55	ND	ND	ND	ND
-OH-4-pentenyl	0.1	ND	ND	ND	ND
-OH-benzyl	ND	ND	ND	ND	ND
-indolymethyl	ND	ND	ND	ND	ND
-OH-3-indolylmethyl	0.80	ND	ND	ND	ND

[1] Values expressed as μmol glucosinolates/g of protein.
[2] ND denotes nondetectable levels.

TABLE 1.17. Glucosinolate content of PMM and acetylated proteins.

INTACT GLUCOSINOLATES	PMM[1]	SAMPLE 16% A-PMM	26% A-PMM	42% A-PMM	62% A-PMM
allyl	ND[2]	ND	ND	ND	ND
-butenyl	0.55	0.1	ND	ND	0.1
-pentenyl	0.30	ND	ND	ND	ND
-OH-3-butenyl	0.55	0.1	0.1	ND	0.1
-OH-4-pentenyl	0.1	ND	ND	ND	ND
-OH-benzyl	ND	ND	ND	ND	ND
-indolymethyl	ND	ND	ND	ND	ND
-OH-3-indolylmethyl	0.80	ND	ND	ND	ND

[1] Values expressed as μmol glucosinolates/g of protein.
[2] ND denotes nondetectable levels.

SUMMARY

In summary, acylation was found to cause extensive changes in protein conformation. Molecular weight decreased as extent of modification increased. This same trend was observed with the enthalpy of denaturation. In addition to changes in protein conformation, the surface charge was also altered, as was evidenced by an overall decrease in the isoelectric points of the acylated proteins. Succinylation resulted in more sizable alterations in protein conformation and surface charge than acetylation. Following acylation, there was a slight decrease in lysine, as well as a decrease in proline following succinylation. The levels of phytic acid and glucosinolates decreased in both the acetylated and succinylated concentrates. Acetylation also caused a decrease in the content of phenolic compounds.

CHAPTER 2

Effects of Acetylation and Succinylation on the Functional Properties of the Canola 12S Globulin

INTRODUCTION

The functional properties of a protein determine its behaviour during food processing, storage and preparation and ultimately govern its suitability as a food ingredient. The critical functional attributes required by a food protein include solubility, heat coagulation, water and fat absorption, gelation, emulsifying properties, whippability, and good sensory properties (Kinsella, 1976). Of these, solubility is the most important, as it allows for ease of distribution of the protein as well as affects its ability to act as a foaming, emulsifying or gelling agent. In cosmetic systems, the most important functional property is substantivity, which refers to the protein's ability to adhere to the skin or hair. Emulsifying and foaming properties are also significant in cosmetic applications.

Many native proteins possess limited functionality; therefore modification, such as acylation, is often performed in order to expand the range of functional properties available. Acylation has been shown to be particularly effective in improving the functional properties related to protein solubility, such as foaming and emulsifying activity (Canella *et al.*, 1979). As well, it has been reported to increase fat absorption (Kabirullah and Wills, 1982) and gelation (Paulson and Tung, 1989). Therefore, proteins with poor functional properties could conceivably be of use in food or cosmetic systems following acylation.

Previous research has identified canola protein isolates and concentrates as having poor functional properties, such as nitrogen solubility, water-holding and gelation (Ponnampalam *et al.*, 1990). In addition, canola protein isolates may be

unacceptably dark due to the presence of phenolic compounds. In other studies, a noticeable lightening of protein isolates has been reported (Choi *et al.*, 1982; Canella *et al.*, 1979) following acylation. Therefore, chemical or enzymatic modification may be required in order to utilize canola proteins as a food or cosmetic ingredient.

The purpose of this study was to determine the effects of acetylation and succinylation on certain functional properties of the canola 12S globulin. Specifically, protein solubililty, foaming and emulsifying properties, fat and water absorption, colour, and gelation were monitored.

MATERIALS AND METHODS

1. Protein Solubility

The protein solubility of the PMM and all acylated proteins were examined in the range of pH 1-9 by the method of Barber and Wartheson (1982) with some modification. Protein samples (1% w/v) were dispersed in 0.1 M NaOH and the pH was adjusted to the desired level with 1 M HCl. After stirring for 1 h, the suspension was centrifuged at 12,000g using a Sorvall Refrigerated Centrifuge, Model RC2-B (DuPont Co., Wilmington, DE), for 20 min at 4°C. The supernatant was filtered through Whatman No. 4 filter paper and the protein content was determined by the Coomassie method (Sedmak and Grossberg, 1977). The percent protein solubility of all samples was expressed as:

$$\% \ Protein \ Solubility = \frac{amount \ of \ protein \ in \ supernatant \times 100}{amount \ of \ protein \ in \ original \ dispersion}$$

2. Emulsifying Properties

The emulsifying properties of PMM and all acylated concentrates were assessed by the method of Yasumatsu *et al.* (1972) with some modification. Protein (0.7 g) was suspended in distilled water (10mL) adjusted to pH 8 with 0.01 M NaOH and 10 mL of corn oil were added. These mixtures were homogenized with an Omnimixer (Ivan Sorvall Inc., Norwalk, CO) for 1 min at a setting of 7. The

emulsion was then divided evenly into two 15 mL centrifuge tubes and centrifuged at 1300g for 5 min. using a Sorvall GLC-1 benchtop centrifuge (Dupont Co., Wilmington, DE). Emulsifying activity was defined as follows:

$$\% \textit{ Emulsifying Activity} = \frac{\textit{height of emulsified layer} \times 100}{\textit{height of the contents of the tube}}$$

Emulsion stability was measured by recentrifugation following heating at 80°C for 30 min and was expressed as:

$$\% \textit{ Emulsion Stability} = \frac{\textit{height of remaining emulsified layer} \times 100}{\textit{height of original emulsified layer}}$$

3. Foaming properties

The procedure of Puski (1975) with some modification was used to assess the foaming properties of PMM and all acylated concentrates. Protein solutions (2% w/v) were prepared in 0.1 M sodium phosphate buffer at pH 8.0. The solutions (50mL) were homogenized for 1 min at a setting of 7 using an Omnimixer. After mixing, the contents were immediately poured into a 100mL graduated cylinder and the volume of the foam layer was recorded. The foaming capacity was defined according to the following equation:

$$\% \textit{ Foam Capacity} = \frac{\textit{foam volume immediately after mixing} \times 100}{\textit{starting volume of liquid phase}}$$

Foam stability was expressed as:

$$\% \ Foam \ Stability = \frac{foam \ volume \ after \ standing \ for \ 1 \ h \times 100}{foam \ volume \ immediately \ after \ mixing}$$

4. Water Holding Capacity

The % water holding capacity (WHC) of all samples was measured according to the method of Thompson *et al.* (1982). A protein sample (0.5g) was mixed with distilled water (3mL) in a 15 mL graduated centrifuge tube. The mixture was stirred occasionally over a period of 30 min, then was centrifuged at 1,760g using a Sorvall GLC-1 centrifuge for 20 min. The supernatant was decanted and the tube was reweighed. The WHC was calculated as follows:

$$\% \ WHC = \frac{weight \ of \ water \ bound \times 100}{weight \ of \ sample}$$

5. Fat Absorption Capacity

The % fat absorption capacity (FAC) was assessed by the procedure of Lin and Humbert (1974). A protein sample (0.3 g) was stirred with corn oil (3 mL) in a preweighed 15 mL graduated centrifuge tube for 1 min. After a holding period of 30 min, the tube was centrifuged for 25 min at 1,760g using a Sorvall GLC-1 centrifuge. The supernatant was decanted and the tube was reweighed. The % FAC was defined as follows:

$$\% \ FAC = \frac{weight \ of \ oil \ bound \times 100}{weight \ of \ sample}$$

6. Colorimetric Determination

The colour of the PMM and acylated concentrates was determined with a Hunterlab Colour Difference Meter D25-2 (Hunter Associates Laboratory Inc., Fairfax, VI). The instrument was calibrated using a standard with values: L=92.37, a=-1.2, and b=0.5. The size of the dish used to obtain the measurements was 10 cm^2.

Sufficient protein concentrate was added to completely cover the bottom of the measuring dish. Three measurements were taken, and the dish was rotated a quarter turn between each measurement.

7. Small Amplitude Oscillatory Rheology

The protein gel formation and rheological properties were characterized using a Bohlin VOR rheometer (Bohlin Reologi, Inc., Lund, Sweden). The rheometer was operated in the small amplitude oscillatory mode using the 30 mm parallel plate geometry. The torque bar was calibrated to 93.1 gcm, and the input strain amplitude for dynamic analysis was 0.02. Approximately 1 mL of a 10% protein dispersion in 0.1 M NaCl was pipetted onto the lower plate. The upper plate was slowly lowered until a gap of 1.0 mm between the two plates was reached. In order to prevent the sample from drying during the heating phase, a perimeter of masking tape was placed around the cylinder supporting the plate to form a well which was filled with mineral oil. Temperature was maintained throughout the analysis by a programmable water bath.

Samples were heated and then cooled over a temperature range of 25-95°C at a rate of 2°C/min at a frequency of 0.10 Hz. Rheological data were collected every 60 s, and at the end of each phase the final temperature was held for 2 min. The frequency sweeps were conducted at a range of 0.01-10.00 Hz at 25°C.

8. Statistical Analysis

All statistical analyses were performed using SAS statistical analysis software program package. Significant differences among treatments were determined by Duncan's Multiple Range test ($p \leq 0.05$).

RESULTS AND DISCUSSION

1. Protein Solubility

The solubility of PMM was determined over the pH range of 1 to 9. The solubility profile of PMM and succinylated proteins is presented in Figure 2.1, and that for PMM and acetylated proteins is presented in Figure 2.2. The values for protein solubility are tabulated in Appendices 7a and 7b, and the analysis of variance for this variable is shown in Appendices 8a through 8i.

The PMM exhibited a solubility curve typical of proteins, namely, decreasing solubility with decreasing pH, and resolubilization at pH values acidic to the isoelectric point. Both succinylation and acetylation impaired the protein's solubility below the isoelectric point. The extent of insolubility increased with an increase in the degree of acylation. The two highest levels of acetylation and succinylation were significantly lower in solubility than the two lowest levels of acylation at pH 1,2 and 3.

Protein solubility was markedly improved at neutral to alkaline pH. At pH 7, PMM was only approximately 25% as soluble as the 3% S-PMM and 48% S-PMM. This trend continued from pH 6 to 9, with the succinylated and acetylated proteins exhibiting significantly higher solubility than the PMM.

Generally the succinylated concentrates were slightly more soluble than the acetylated concentrates. In most cases, the two lower levels of acylation were equally as effective in enhancing protein solubility as the two higher levels, indicating that extensive acylation was not required to improve solubility. This trend was also observed by Ponnampalam *et al.* (1990) with rapeseed proteins and Sheen (1991) with tobacco leaf proteins.

FIGURE 2.1. Solubility profile of PMM and succinylated concentrates.

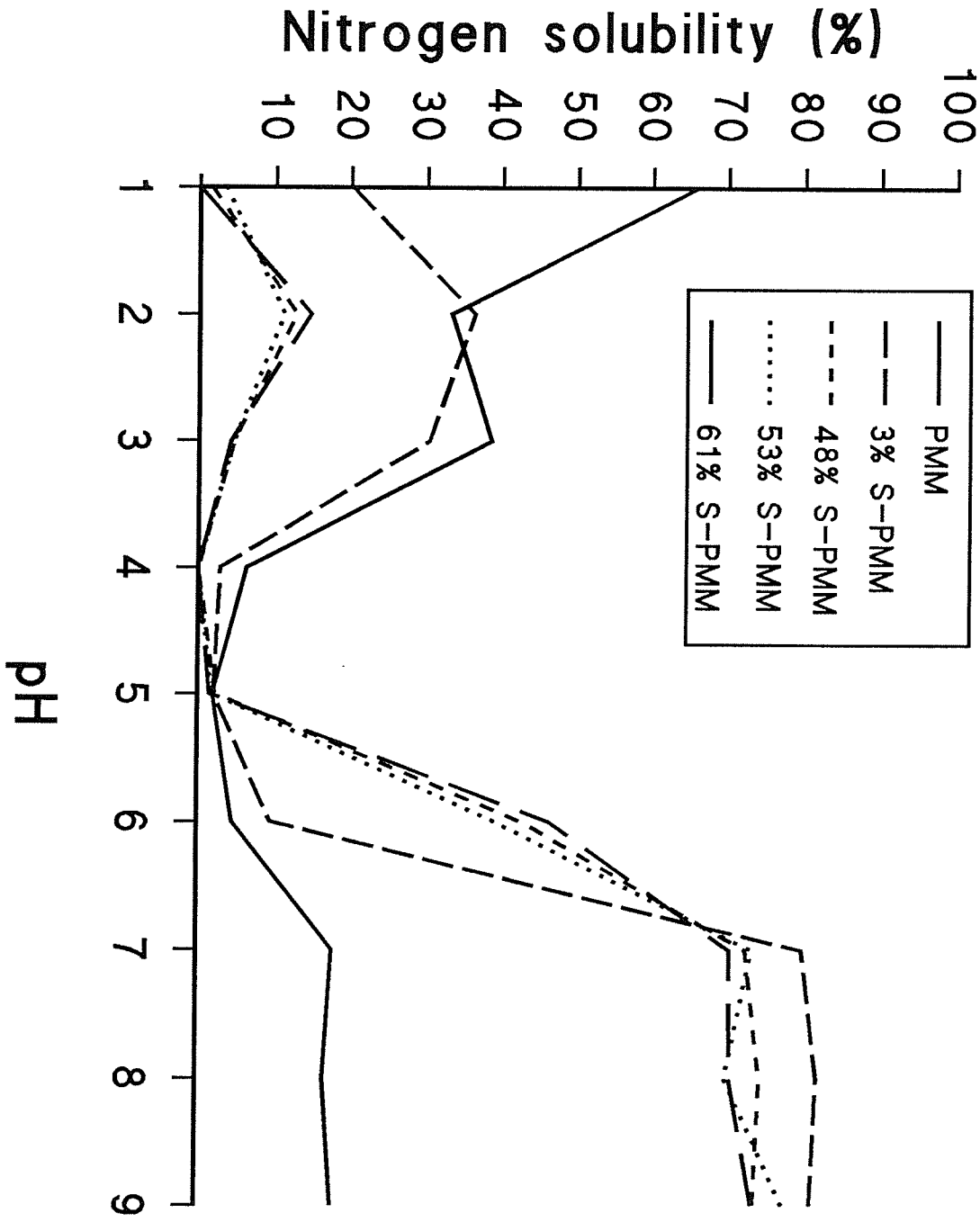
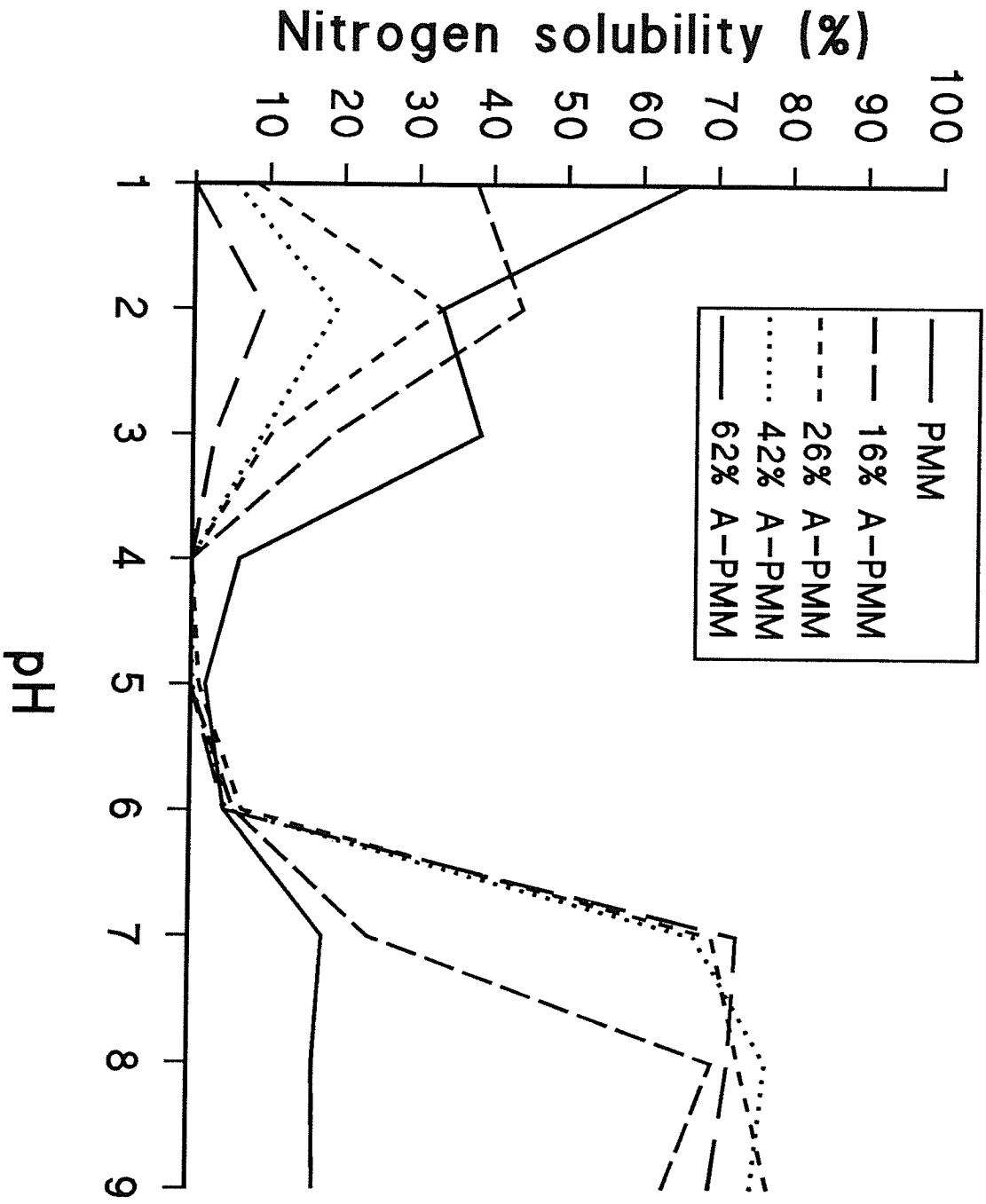


FIGURE 2.2. Solubility profile of PMM and acetylated concentrates.



Impaired solubility below the isoelectric point has also been reported by Barber and Wartheson (1982) with wheat gluten and Franzen and Kinsella (1976a) with soy proteins. Franzen and Kinsella (1976a) suggested that this effect is due to the removal of cationic ammonium groups from lysine by acylation resulting in an insufficient number of hydrophilic cationic groups to exceed the aggregate forces resulting from hydrophobic interactions between the alkyl and aromatic groups of constituent amino acid residues.

Increased solubility at neutral to alkaline pH has been observed by others in the acylated forms of corn germ protein isolate (Messinger *et al.*, 1987), wheat gluten (Barber and Wartheson, 1982) and sunflower proteins (Kabirullah and Wills, 1982). Following the acylation of canola proteins, Paulson and Tung (1987) attributed this to the combination of intra- and intermolecular charge repulsion promoting protein unfolding and producing fewer protein-protein interactions and more protein-water interactions.

2. Emulsifying Properties

The role of proteins in contributing to the formation and stabilization of emulsions is critical for many applications in the food and cosmetic industry. Emulsions of oil and water are thermodynamically unstable due to the positive free energy caused by interfacial tension. The stabilization of the emulsion droplets by proteins is achieved by the formation of a film around the droplets, which consequently lowers interfacial tension and physically prevents coalescence. The ability of a protein to lower interfacial tension and form a film is a function of the ease with which it can migrate to, adsorb at, unfold and rearrange at an interface (German *et al.*, 1985). This is related to several structural features of the protein, including flexibility, stability of the native conformation, and surface hydrophobicity.

The emulsifying activity of PMM was found to be 39.8% (Figure 2.3). The 3%, 48% and 54% S-PMM showed a significant increase in emulsifying activity in comparison with PMM. The 61% S-PMM had an emulsifying capacity of 100%, which was significantly greater than all the other levels of succinylation and acetylation as well as the PMM. For the acetylated proteins, the emulsifying activity increased almost twofold at the 16% level, then decreased slightly at the two intermediate levels. The highest level of acetylation was not significantly greater than the 16% A-PMM.

The emulsion stability of the PMM was found to be 68.0% (Figure 2.4). The 3% S-PMM had an emulsion stability of 96.7%, which then decreased as the level of succinylation increased. The 61% S-PMM had an emulsion stability

FIGURE 2.3. Emulsifying activity of PMM and all acylated concentrates.

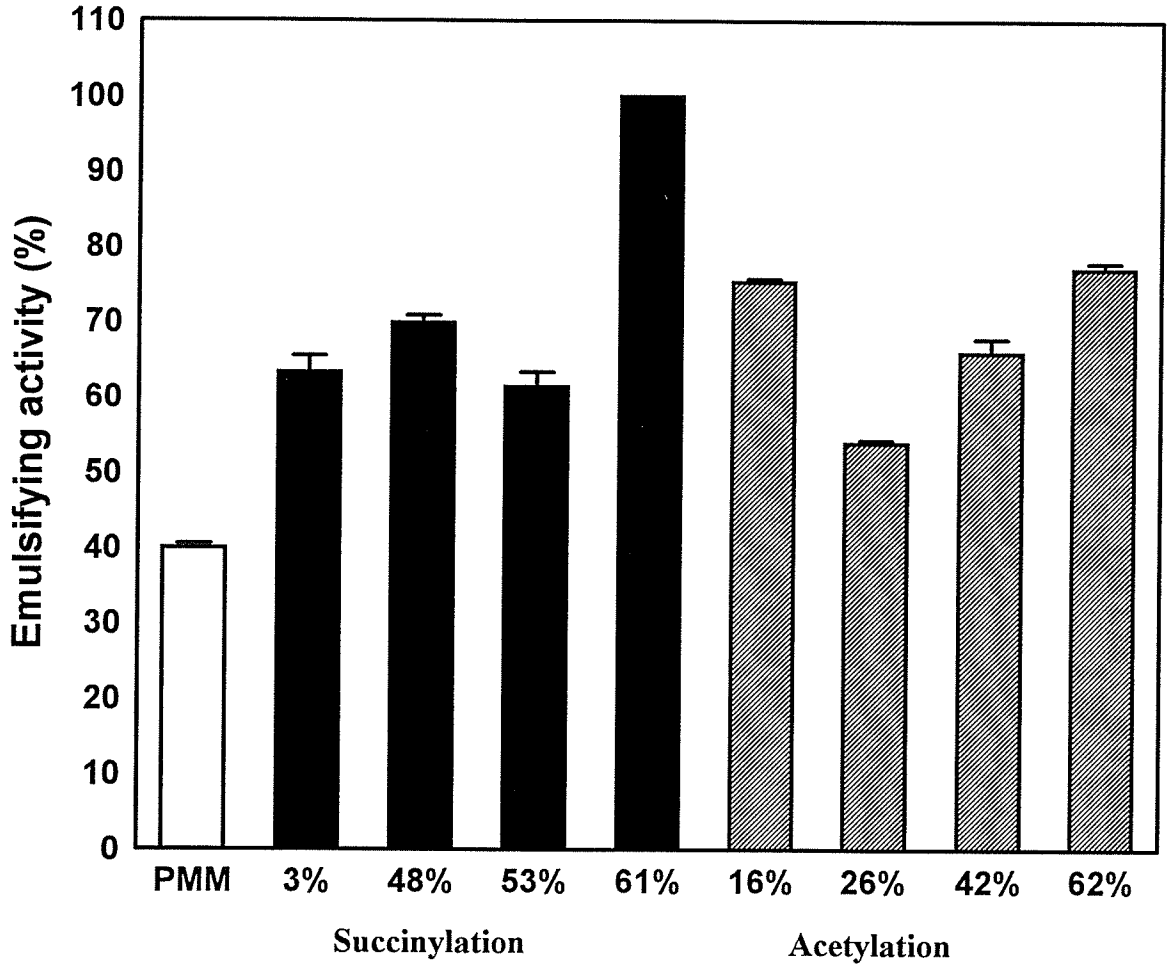
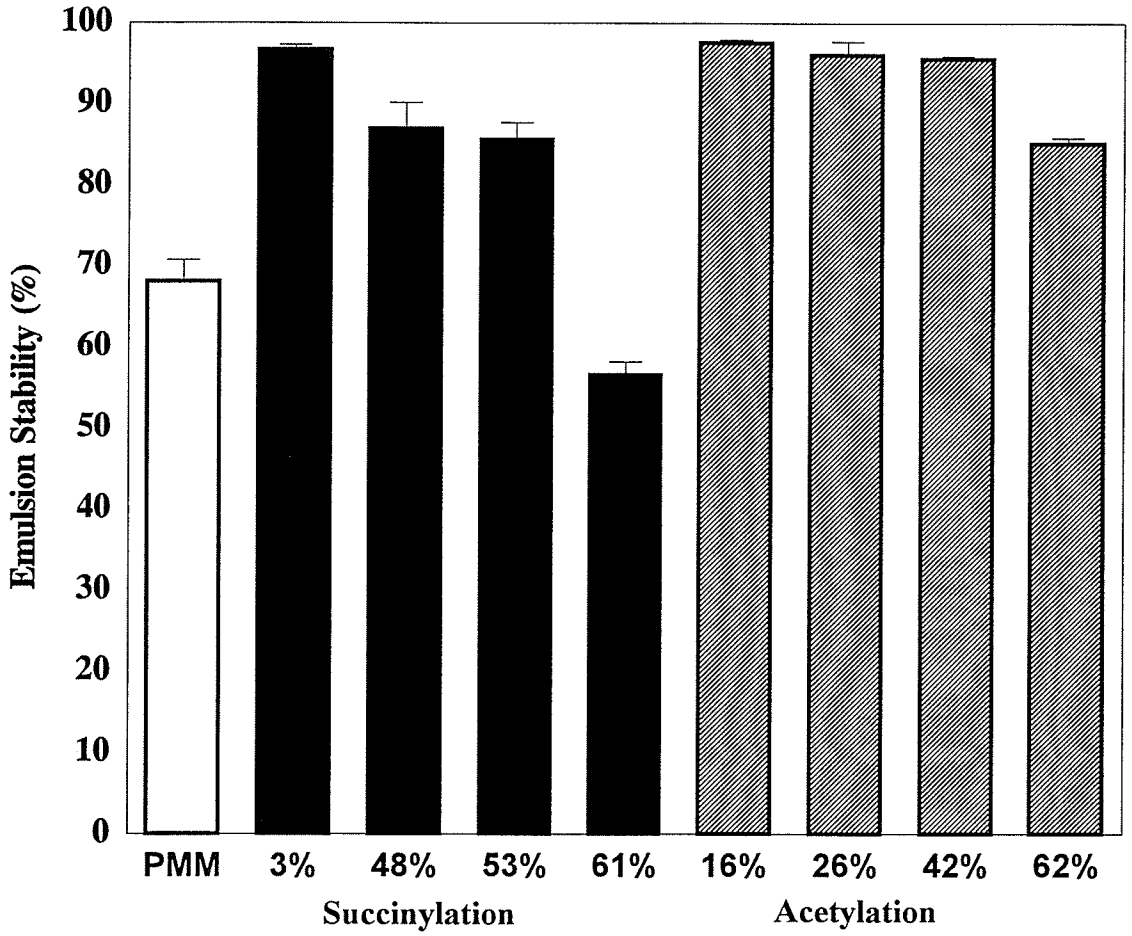


FIGURE 2.4. Emulsion stability of PMM and all acylated concentrates.



of 56.4%, which was significantly lower than the PMM. The acetylated protein concentrates exhibited a similar pattern, only the decrease in emulsifying stability was not as steep as was observed with the succinylated samples, as they were all significantly higher than the PMM. The values for emulsifying activity and emulsifying stability are given in Appendix 9, the analysis of variance is presented in Appendices 10a and 10b.

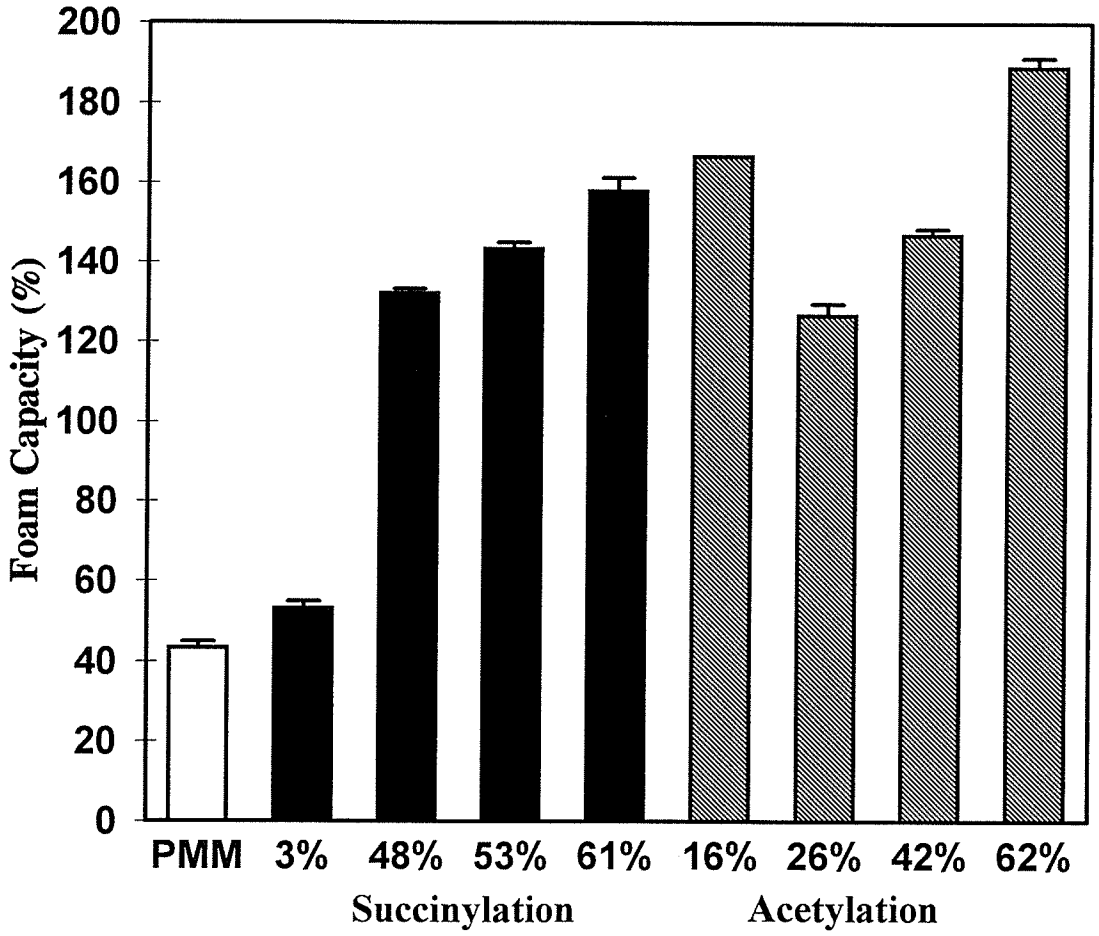
The dramatic increase in emulsifying activity following acylation may be a reflection of the increased solubility and more open structure which facilitated diffusion of the protein to and rearrangement at the interface. Insoluble protein does not play a significant role in emulsifying properties as it must dissolve and migrate to the interface before it can contribute. In addition, Kato and Nakai (1980) reported a correlation between surface hydrophobicity and emulsifying activity of proteins. Molecular flexibility is also an important factor in interfacial properties. Kim and Rhee (1989) state that molecular flexibility is inversely proportional to enthalpy. The enthalpy of the acylated proteins decreased with increasing modification, as was evidenced by differential scanning calorimetry (Chapter 1). Therefore, the increase in molecular flexibility may contribute to the improvement in emulsifying properties. Paulson and Tung (1988) investigated the emulsifying properties of canola protein isolates, and reported an increase in emulsifying activity and emulsifying stability following succinylation. Similar results were reported by Ma and Wood (1987) and Childs and Park (1976) with oat proteins and cottonseed flour, respectively.

3. Foaming properties

A foam may be defined as a two-phase system in which a distinct gas bubble phase is surrounded by a continuous liquid lamellar phase (German *et al.*, 1985). The function of a food or cosmetic protein in a foam is to lower the energy or tension at the gas-liquid interface. Therefore, for a protein to be a good foaming agent it must be soluble, small and flexible enough to rapidly absorb to and coat fresh surfaces, and it must be able to form a stable film through intermolecular interactions with adjacent molecules (Thompson and Cho, 1984). These properties depend upon the viscosity, hydrophobicity, solubility and net charge density of the protein (Nakai, 1983).

The PMM had a foaming capacity of 43.3% (Figure 2.5). Succinylation caused an increase in foaming capacity, with all the levels being significantly different from each other as well as the PMM (Appendix 11). The highest value of foaming capacity for the succinylated concentrates was 157.8% for the 61% S-PMM. The acetylated protein concentrates all had significantly higher foaming capacities then the PMM. However, the foaming capacities at the 26% and 42% levels of acetylation was lower than that at the 16% level. An increase in foaming capacity with acylation at similar levels of modification has been frequently cited by other researchers, including Ma (1984) with oat proteins, Messinger *et al.* (1987) with corn germ protein isolates, and Franzen and Kinsella (1976b) with leaf proteins. This increase in foaming capacity may be due to a number of factors. Firstly, protein solubility has been found to make an important contribution to foaming behaviour (Hermansson, 1973). The reduction in molecular size of the acylated proteins, as evidenced by gradient PAGE (Chapter 1)

FIGURE 2.5. Foaming capacity for PMM and all acylated concentrates.

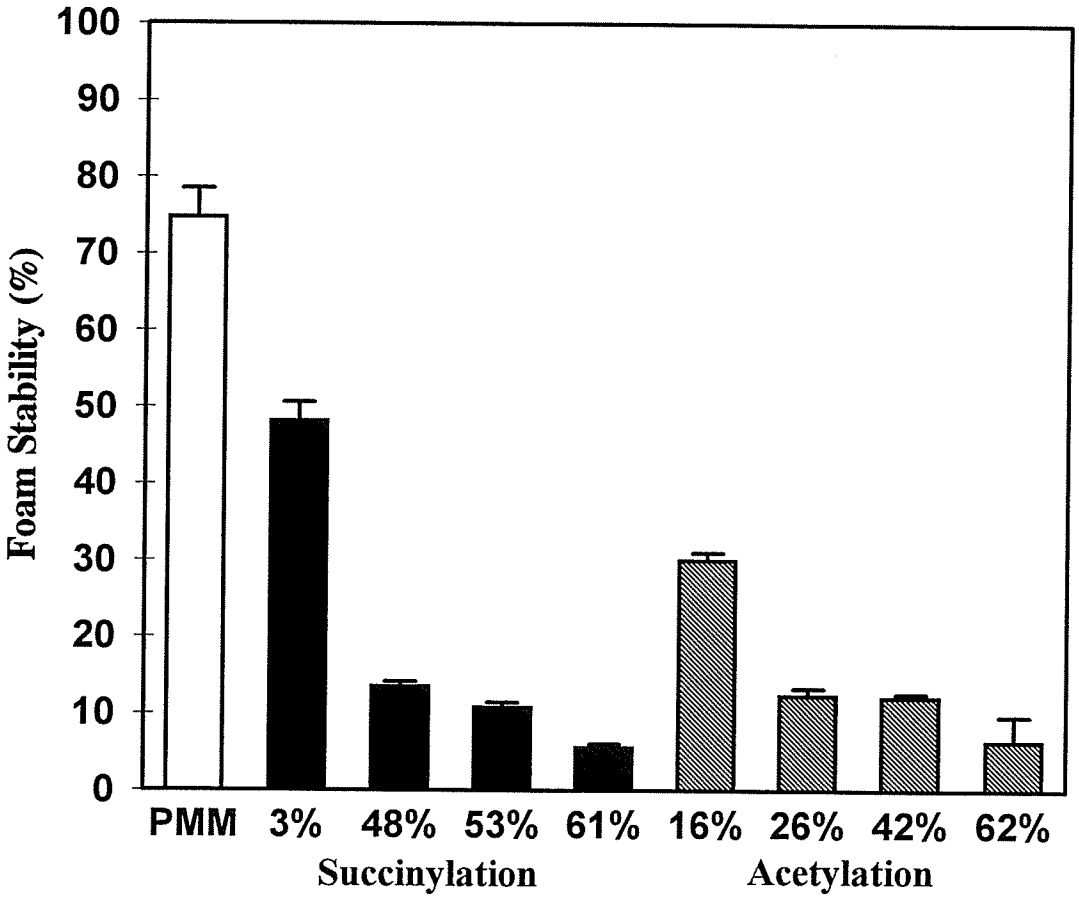


would enable them to move more quickly to the air-water interface. In addition, dissociation of the protein would increase its molecular flexibility, allowing it to unfold more easily at the interface. The higher foaming capacities of the acetylated proteins as compared to the succinylated proteins may be attributed to the higher surface hydrophobicity of the acetylated proteins. Previous research by Townsend and Nakai (1983) has shown a strong correlation between the surface hydrophobicity of various proteins and their ability to produce foams.

The foam stability of PMM was found to be 74.7% (Figure 2.6). The succinylated protein concentrates showed a significant decrease in foam stability with increasing modification, to a low of 5.6% with the 61% S-PMM. The acetylated proteins followed a similar trend. Ma (1984) plus Thompson and Cho (1984) also reported a decrease in foam stability following acylation of oat proteins and rapeseed proteins, respectively. The decrease in foam stability is a result of the increased charge density of acylated proteins, since it inhibits the protein-protein interactions which are necessary to form a continuous network around the air bubbles (Townsend and Nakai, 1983).

The values for foaming capacity and foam stability are presented in Appendix 11, and the analysis of variance for these two variables is shown in Appendices 12a and 12b.

FIGURE 2.6. Foam stability for PMM and all acylated concentrates.



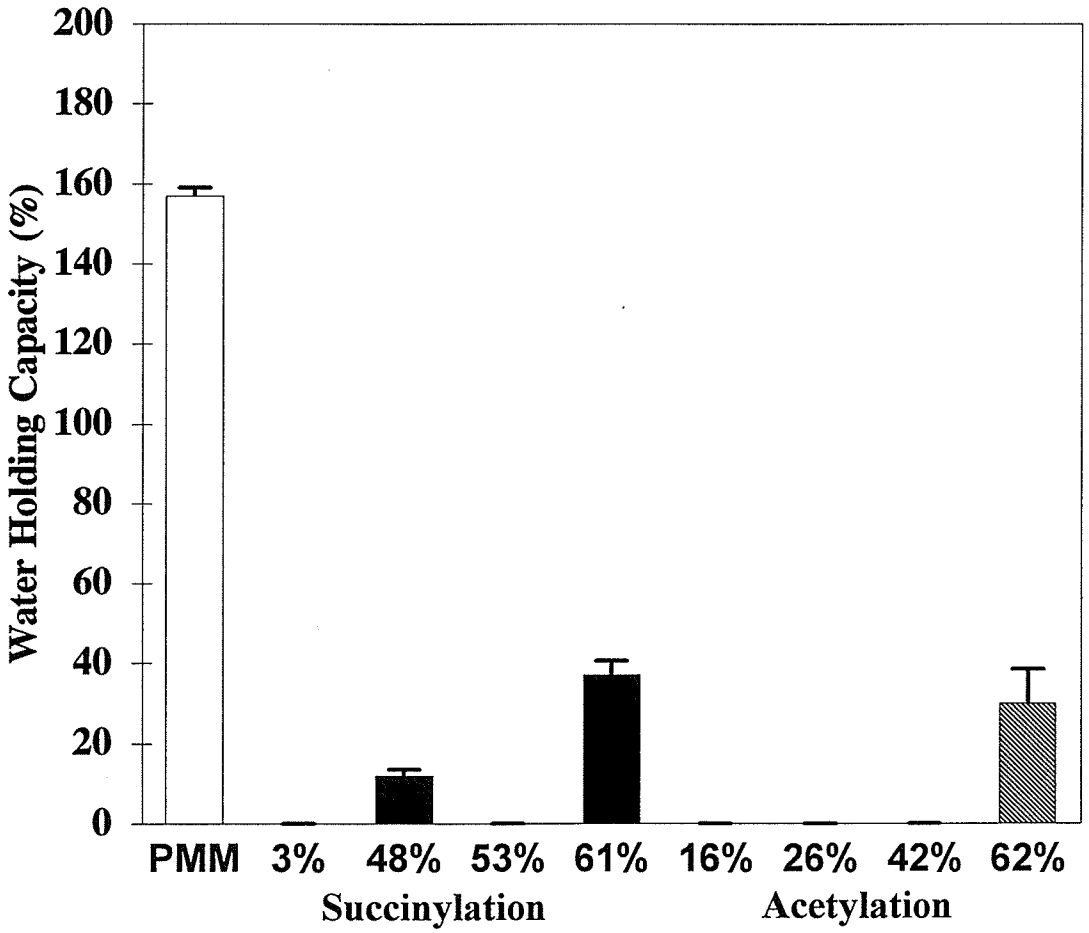
4. Water holding capacity

Water holding, or water binding, connotes the water retained (bound and entrapped) by the protein after centrifugation, and represents the ability of the protein to physically hold water against gravity (Kinsella, 1977).

The water holding capacity (WHC) of PMM was found to be 157% (Figure 2.7). Upon acylation of the protein, the WHC decreased dramatically, going to zero values at 3% S-PMM, 53% S-PMM, 16% A-PMM, 26% A-PMM and 42% A-PMM. The 61% S-PMM and 62% A-PMM retained some WHC, but this was still a four fold decrease from the value observed in the PMM. The values for water holding capacity are presented in Appendix 13, and the analysis of variance is given in Appendix 14.

Ma (1984) investigated the water absorption properties of oat protein and also reported a decrease in WHC upon modification. This was attributed to the increased solubility of the protein, as Hermansson (1973) had previously determined that highly soluble protein exhibits poor water absorption. Barman *et al.* (1977) reported a decrease in the water binding capacity of acetylated soy protein isolates, as determined by NMR, and this was attributed to the elimination of the charged ε-amino groups of lysine.

FIGURE 2.7. Water holding capacity of PMM and all acylated concentrates.

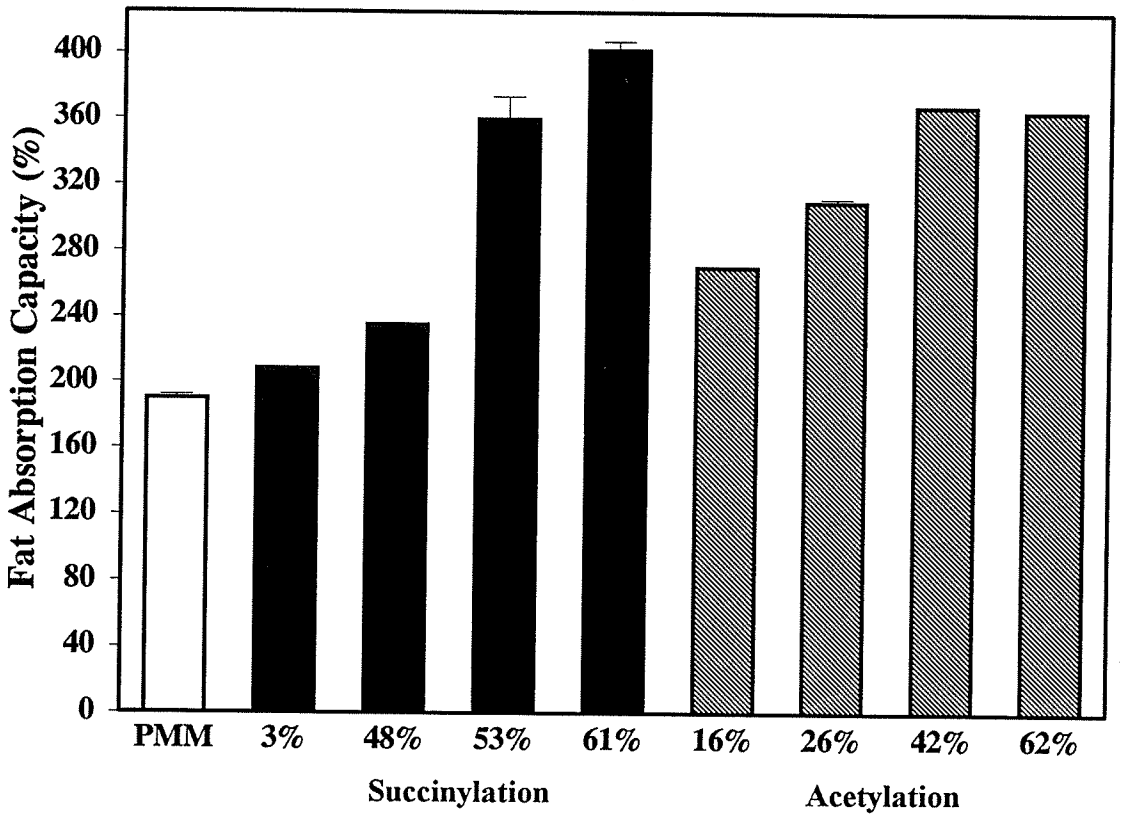


5. Fat absorption capacity

PMM had a fat absorption capacity (FAC) of 190% (Figure 2.8). With the exception of the 3% S-PMM, the FAC of the succinylated protein concentrates were all significantly higher than the PMM, and there was a significant increase with each level of succinylation. The 61% S-PMM had an FAC of 402%, which is a twofold increase over the PMM. A similar pattern was observed with the acetylated protein concentrates, but there was not a significant difference between the 42% A-PMM and 62% A-PMM. The values for fat absorption capacity are tabulated in Appendix 15, and the analysis of variance is presented in Appendix 16.

According to Kinsella (1976), the mechanism of fat absorption has been attributed mostly to the physical entrapment of oil, but as well may be influenced by hydrophobicity. Acylation causes a marked decrease in the bulk density of proteins as a result of unfolding of the polypeptides. Thompson and Cho (1984) were able to correlate the fat absorption capacity of oat proteins with the bulk density. Therefore, the increase in the fat absorption capacity of the acylated proteins in this study may be contributed to the more open structure which allows for physical entrapment of the oil. Furthermore, the higher surface hydrophobicity of the acetylated proteins may also augment the fat absorption capacity. Ma and Wood (1987) plus Kabirullah and Wills (1982) also reported an increase in fat absorption capacity following acylation of oat proteins and sunflower proteins respectively.

FIGURE 2.8. Fat absorption capacity of PMM and all acylated concentrates.



6. Colour determination

The L value of PMM was found to be 48.7 (Table 2.1). All of the acylated concentrates had significantly higher L values. The 16% A-PMM had the highest L value of 67.6. An increase in the L value indicates an increase in lightness of the protein concentrates. In addition to the increase in L values, there was a significant decrease in a and b values following acylation. The decrease in the a and b values of the acylated concentrates is indicative of a loss of colour. The analysis of variance of the L, a, and b values is presented in Appendix 17a, Appendix 17b, and Appendix 17c respectively.

Choi *et al.* (1982) and Canella *et al.* (1979) reported isolates that were lighter in colour as a result of acylation of cottonseed and sunflower proteins respectively.

TABLE 2.1. Hunterlab determination values of PMM and all acylated concentrates[1].

Sample	L[2]	a[2]	b[2]
PMM	48.7 ± 0.5^{f}	2.5 ± 0.1^{a}	18.8 ± 0.2^{a}
3% S-PMM	63.8 ± 0.3^{d}	0.3 ± 0.1^{c}	16.2 ± 0.2^{cd}
48% S-PMM	62.1 ± 0.3^{e}	0.1 ± 0^{d}	16.4 ± 0.1^{c}
53% S-PMM	63.9 ± 0.2^{d}	0.0 ± 0^{de}	16.0 ± 0^{d}
61% S-PMM	64.4 ± 0.1^{c}	0.0 ± 0^{de}	16.0 ± 0.2^{d}
16% A-PMM	67.6 ± 0.1^{a}	0.0 ± 0^{de}	17.4 ± 0.1^{b}
26% A-PMM	63.9 ± 0.2^{d}	-0.2 ± 0.1^{f}	17.4 ± 0.1^{b}
42% A-PMM	62.3 ± 0.1^{e}	0.5 ± 0.1^{b}	16.4 ± 0.1^{c}
62% A-PMM	65.4 ± 0.3^{b}	-0.1 ± 0^{ef}	16.4 ± 0^{c}

[1] Each value is a mean of three determinations.

[2] Values followed by the same letter are not significantly different ($p \leq 0.05$).

7. Gelation properties

A gel may be defined as a well hydrated insoluble three-dimensional network in which the protein-protein and protein-solvent interactions occur in an ordered manner (Cheftel *et al.*, 1985). The mechanism of gelation of globular proteins is most commonly accepted to follow the two-step mechanism proposed by Ferry (1948). Initially, the protein molecules unfold or dissociate, which is followed by aggregation and association; this action, under appropriate thermodynamic conditions, results in the formation of a gel. The bonds within protein gels vary with both the type of protein and the gelation environment, and may include hydrophobic interactions, ionic attractions, hydrogen bonds, and disulfide linkages (Paulson and Tung, 1989).

The parameters of protein network formation which are most often examined by dynamic rheology are the storage modulus (G'), loss modulus (G") and tan δ (G"/G'). The G' relates to the elastic nature of the material, the G" relates to the viscous nature of the material, and the tan δ denotes the relative energy from the viscous and elastic components. Of these, the tan δ has been found to be the most useful for determining the contribution of G' and G" to the final gel structure (Arntfield, 1989). Arntfield (1989) reported that a low tan δ in ovalbumin gels indicated a well cross-linked network, while a high tan δ was indicative of an aggregated structure.

The G' of PMM was found to be 12.12 (Table 2.2). This value was significantly lower than that of all of the acylated concentrates with the exception of

the 61% S-PMM. The highest G' was 914.5 with the 42% A-PMM, which was significantly greater than any of the other samples. The G" of PMM was 2.86. The G" values of the 61% S-PMM and the 48% S-PMM were not significantly different from the PMM, but all the other samples were significantly higher than the PMM. As with the values for G', the 46% A-PMM had a significantly higher G" than all of the other samples. The PMM had a tan δ of 0.233. The 48% S-PMM, 53% S-PMM and 61% S-PMM had a significantly lower tan δ than all other samples, indicating better cross-linked networks. The analysis of variance of G', G" and tan δ are presented in Appendix 18a, Appendix 18b and Appendix 18c respectively.

Table 2.2. Gelation properties of PMM and all acylated concentrates as determined by dynamic rheology[1].

Sample	G'[2]	G"[2]	Tan δ[2]
PMM	12.12 ± 9.03^{e}	2.86 ± 2.20^{e}	0.238 ± 0.001^{a}
3% S-PMM	395.0 ± 62.2^{bc}	54.05 ± 9.26^{bc}	0.137 ± 0.002^{b}
48% S-PMM	235.0 ± 29.7^{d}	19.8 ± 2.26^{de}	0.085 ± 0.001^{c}
53% S-PMM	462.5 ± 45.96^{b}	36.65 ± 5.44^{cd}	0.079 ± 0.004^{c}
61% S-PMM	27.4 ± 7.57^{e}	1.67 ± 0.52^{e}	0.061 ± 0.002^{c}
16% A-PMM	314.0 ± 41.0^{dc}	51.9 ± 7.00^{bc}	0.166 ± 0.001^{b}
26% A-PMM	479.5 ± 79.9^{b}	74.9 ± 11.81^{b}	0.156 ± 0.004^{b}
42% A-PMM	914.5 ± 62.9^{a}	149.0 ± 22.6^{a}	0.163 ± 0.013^{b}
62% A-PMM	300.0 ± 2.830^{dc}	60.8 ± 12.3^{bc}	0.203 ± 0.042^{a}

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value is the mean of two determinations.

The increase in G' and G" signify an increase in gel strength, and in the protein-protein interactions contributing to both the G' and G" components of the network. However, the significantly higher tan δ values resulting from acetylation as compared to succinylation would indicate that the protein-protein interactions contributing to G" were greater in the acetylated concentrates. Paulson and Tung (1988) tentatively identified the bonds involved in gel formation and stability of canola proteins to be hydrophobic interactions and hydrogen bonding. Therefore, the higher G' and G" of the acetylated concentrates may be attributed to their higher surface hydrophobicity (Chapter 1). Paulson and Tung (1989) reported an increase in the G' and G" of the gels of succinylated canola protein isolates. These values were the highest at intermediate levels of modification. Ma and Wood (1987) reported an increase in gel strength of oat proteins following acylation at similar levels of modification as in this study.

SUMMARY

In summary, acylation appeared to be effective in enhancing several functional properties of proteins. Specifically, protein solubility at neutral to alkaline pH levels, emulsifying properties, foaming capacity, fat absorption capacity and gelation were markedly improved. In addition, the acylated concentrates were lighter in colour than the PMM. However, acylation was found to be detrimental to water holding capacity and foam stability.

GENERAL SUMMARY AND CONCLUSIONS

The main objective of this study was to modify PMM through acetylation and succinylation to obtain a canola protein concentrate with improved functional properties. In addition to functionality, several physicochemical characteristics and levels of antinutritional factors of the acylated concentrates were investigated. The acylated concentrates were found to decrease in molecular weight with increasing modification, indicating dissociation of the protein structure. Acetylation did not cause as extensive unfolding and dissociation as succinylation, as was evidenced by a less of a decrease in molecular weight. At the 62% level of succinylation, only a band corresponding to the 2-3S subunit was present, indicating complete dissociation of the protein into subunits. In addition to a decrease in molecular weight, the acylated concentrates exhibited isoelectric points that were lower than the value of 7.1 determined for the PMM. The acetylated concentrates had isoelectric points in the range of 4.9-6.1, and the succinylated concentrates had isoelectric points in the range of 4.6-5.5. Denaturation of the acylated concentrates was also illustrated by a decrease in the enthalpy of the protein as the level of modification increased. The decrease in enthalpy was more pronounced with the succinylated proteins than with the acetylated proteins. The temperature of denaturation decreased upon succinylation, but increased upon acetylation. Furthermore, extensive changes in protein conformation were determined by alterations in the UV and fluorescence spectra of the acylated proteins. Determination of the surface hydrophobicity indicated an increase in aromatic and aliphatic hydrophobicity of the acetylated

proteins. In contrast, the aromatic hydrophobicity of the succinylated proteins increased, while the aliphatic hydrophobicity decreased. The amino acid profile remained unchanged with the exception of a slight decrease in lysine in both the succinylated and acetylated concentrates, and a decrease in proline in the succinylated concentrates.

Following acylation, there was an overall decrease in the levels of phytic acid and glucosinolates in the modified concentrates as compared to the PMM. Acetylation also caused a decrease in the levels of phenolics. These effects would result in improvements in colour, flavour and nutritional value.

In terms of functionality, acylation resulted in significant changes in the solubility profile of PMM. Generally, solubility below the isoelectric point was impaired by acylation, but solubility at neutral and alkaline pH values was greatly enhanced. The foaming capacity was improved by both acetylation and succinylation, with the 62% S-PMM exhibiting the highest foaming capacity. Foam stability was found to decrease steadily with increasing modification. Emulsifying activity also increased as a result of acylation, up to a value of 100% for the 61% S-PMM. The emulsion stability increased with the first three levels of modification, then declined at the highest level of both acetylation and succinylation. The water holding capacity decreased dramatically for all of the acylated concentrates. However, the fat absorption capacity was elevated by acylation. The gel strength of the succinylated and acetylated concentrates initially increased upon modification, as was evidenced by an increase in G' and G" values. At the 61% S-PMM and 62% A-

PMM, the gel strength decreased. The succinylated concentrates had lower tan δ values than the acetylated concentrates, indicating a well cross-linked protein network was formed by the succinylated samples. All of the acylated concentrates were lighter in colour than the PMM.

Optimal functional properties were obtained at various levels of modification. Protein solubility exhibited the greatest improvements at low to intermediate levels of acylation. In contrast, foam capacity and emulsifying activity were best at the highest level of acetylation and succinylation, as a low molecular weight and unfolded structure are beneficial for good interfacial activity. Emulsion and foam stability, however, decreased at high levels of modification, indicating that some globular structure is required to maintain a stable foam or emulsion. Fat absorption capacity was also highest as degree of modification, and therefore denaturation increased. Gel strength and network formation were greatest with a slightly denatured structure at intermediate levels of acetylation and succinylation.

Overall, the improved functionality and lighter colour of the acylated concentrates would make them suitable for both food and cosmetic applications. Protein solubility at neutral pH values would allow for incorporation of the concentrates into low acid foods and most cosmetic systems. In particular, acylated canola protein concentrates would be beneficial for those applications requiring a foaming, emulsifying or fat absorbing agent.

To further extend this study, the functional properties of acylated canola protein concentrates could be examined at varying pH levels and ionic environments. This would allow for a more thorough knowledge of the possible applications of canola proteins. Furthermore, the behaviour of acylated concentrates in model food and cosmetic systems may be examined.

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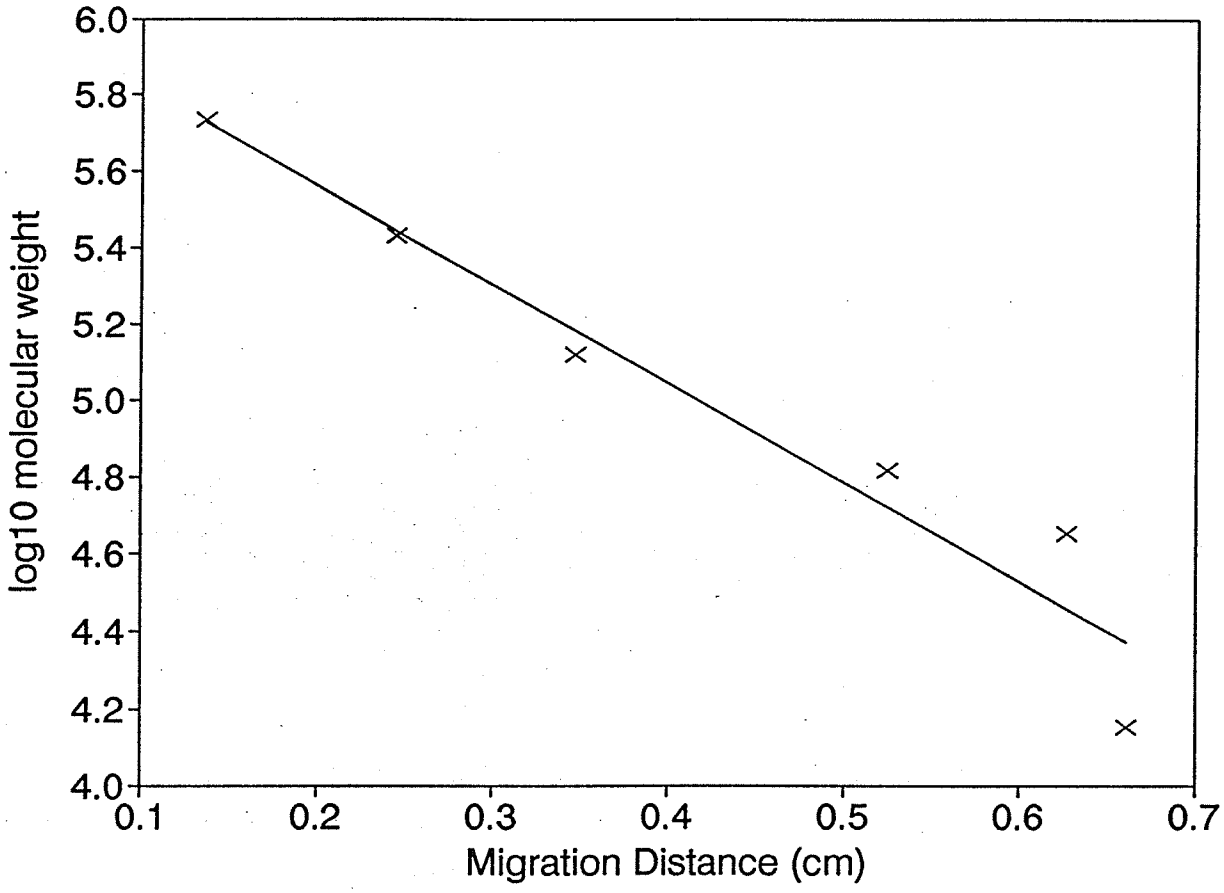
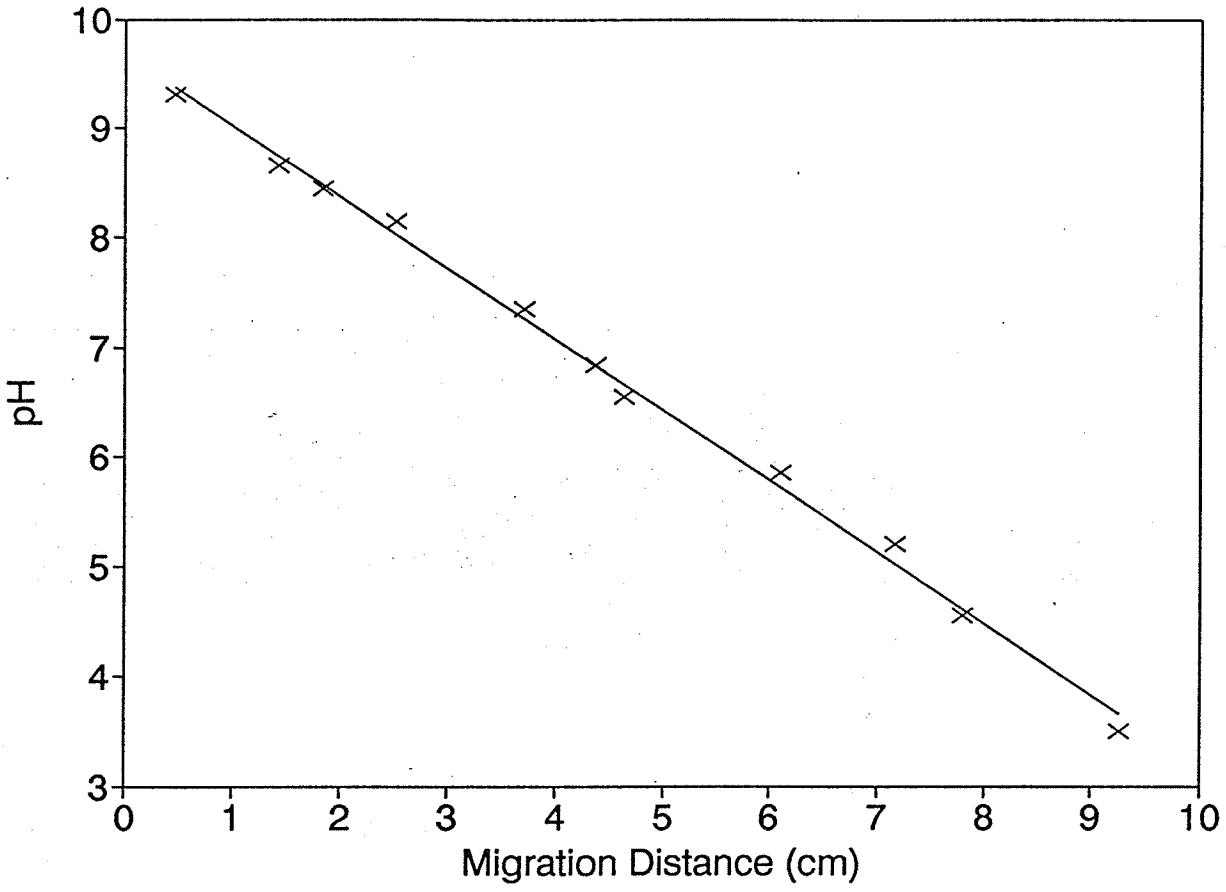
APPENDICES

APPENDIX 1. Calibration curve for molecular weight determination of PMM and all acylated concentrates by gradient PAGE.



APPENDIX 2. Calibration curve for isoelectric point determination of PMM and all acylated concentrates by isoelectric focusing.



APPENDIX 3a. Analysis of variance of temperature of denaturation (Td).

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	35454.705	3545.470	63037.33	0.0001
Error	16	0.900	0.056		
Corrected Total	12	635455.604			

R-Square	C.V.Root	MSE	Td Mean
0.999975	0.350283	0.2372	67.705

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	35454.702	4431.838	78796.66	0.0001
REP	2	0.003	0.001	0.02	0.9758

APPENDIX 3b. Analysis of variance of enthalpy of denaturation (ΔH).

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	455.37094	45.53709	209.13	0.0001
Error	16	3.48395	0.21775		
Corrected Total	26	458.85489			

R-Square	C.V.	Root MSE	ΔH Mean
0.992407	9.094142	0.4666	5.1311

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	455.20463	56.90058	261.32	0.0001
REP	2	0.16631	0.08316	0.38	0.6886

APPENDIX 4a. Analysis of variance of aromatic surface hydrophobicity.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	164453.65	16445.37	2552.03	0.0001
Error	16	103.10	6.44		
Corrected Total	26	164556.76			

R-Square	C.V.	Root MSE	So Mean
0.999373	2.380491	2.5385	106.64

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	164447.36	20555.92	3189.91	0.0001
REP	2	6.29	3.15	0.49	0.6226

APPENDIX 4b. Analysis of variance of aliphatic surface hydrophobicity.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	136687.10	13668.71	76.04	0.0001
Error	16	2876.10	179.76		
Corrected Total	26	139563.20			

R-Square	C.V.	Root MSE	So Mean
0.979392	5.654610	13.407	237.10

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	136572.00	17071.50	94.97	0.0001
REP	2	115.10	57.55	0.32	0.7306

APPENDIX 5. Analysis of variance of phytic acid content.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	1.5772000	0.1752444	18.28	0.0002
Error	8	0.0767111	0.0095889		
Corrected Total	17	1.6539111			

R-Square	C.V.	Root MSE	Mean
0.953618	7.911184	0.0979	1.2378

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	1.5633111	0.1954139	20.38	0.0001
REP	1	0.0138889	0.0138889	1.45	0.2632

APPENDIX 6. Analysis of variance of phenolic content.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	3.5223047	0.3913672	219.72	0.0001
Error	8	0.0142498	0.0017812		
Corrected Total	17	3.5365545			

R-Square	C.V.	Root MSE	Mean
0.995971	5.887635	0.0422	0.7168

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	3.5184620	0.4398078	246.91	0.0001
REP	1	0.0038427	0.0038427	2.16	0.1801

APPENDIX 7a. Protein solubility of PMM and all acylated concentrates in the pH range of 1 to 5[1,2].

Sample	pH 1	2	3	4	5
PMM	65.9± 2.4^{a}	33.3 ± 0.22^{c}	38.6 ± 1.2^{a}	6.37 ± 0.22^{a}	1.95 ± 0.06^{ab}
3% PMM	20.2 ± 4.4^{c}	36.4 ± 0.93^{b}	30.4 ± 0.53^{b}	2.89 ± 0.16^{b}	2.08 ± 0.72^{ab}
48% S-PMM	1.58 ± 0.10^{ef}	12.8 ± 0.11^{ef}	4.67 ± 1.2^{e}	0^{c}	2.28 ± 0.35^{a}
53% S-PMM	3.44 ± 0.30^{def}	11.2 ± 1.9^{fg}	4.63 ± 1.6^{e}	0^{c}	1.79 ± 0.76^{ab}
61% S-PMM	0^{f}	14.7 ± 0.13^{e}	4.19 ± 1.1^{e}	0^{c}	1.52 ± 0.58^{ab}
16% A-PMM	37.8 ± 4.6^{b}	44.0 ± 4.3^{a}	18.7 ± 0.69^{c}	0^{c}	0^{d}
26% A-PMM	8.23 ± 0.33^{d}	32.2 ± 2.1^{c}	10.5 ± 0.54^{d}	0^{c}	1.10 ± 0.42^{bc}
42% A-PMM	5.42 ± 1.2^{de}	19.2 ± 1.3^{d}	9.35 ± 0.10^{d}	0^{c}	0.230 ± 0.020^{cd}
62% A-PMM	0^{f}	9.36 ± 0.02^{g}	3.00 ± 0.19^{e}	0^{c}	0^{d}

[1] Each value represents the mean of two determinations.
[2] Samples followed by the same letter are not significantly different (p≤0.05).

APPENDIX 7b. Protein solubility of PMM and all acylated concentrates in the pH range of 6 to 9[1,2].

Sample	pH 6	7	8	9
PMM	4.57 ± 0.11^{b}	17.9 ± 1.03^{c}	16.8 ± 0.77^{c}	18.6 ± 0.86^{d}
3% S-PMM	9.60 ± 1.9^{b}	80.0 ± 7.1^{a}	82.5 ± 3.8^{a}	81.3 ± 3.2^{a}
48% S-PMM	42.9 ± 8.1^{a}	72.7 ± 2.9^{b}	74.6 ± 1.5^{ab}	73.9 ± 2.4^{ab}
53% S-PMM	39.4 ± 2.14^{a}	73.5 ± 0.61^{ab}	70.1 ± 1.5^{b}	77.7 ± 1.7^{ab}
61% S-PMM	46.5 ± 11.9^{a}	70.6 ± 1.9^{b}	70.7 ± 1.1^{b}	73.7 ± 3.3^{ab}
16%A-PMM	5.88 ± 0.30^{b}	24.0 ± 0.01^{c}	70.1 ± 4.4^{b}	63.7 ± 1.9^{c}
26% A-PMM	6.99 ± 0.25^{b}	70.0 ± 5.6^{b}	73.5 ± 8.6^{b}	77.8 ± 6.8^{ab}
42% A-PMM	4.89 ± 1.4^{b}	73.5 ± 8.6^{b}	77.3 ± 2.2^{ab}	75.5 ± 1.4^{ab}
62% A-PMM	4.51 ± 0.58^{b}	77.8 ± 6.8^{ab}	75.5 ± 1.4^{b}	69.8 ± 7.0^{bc}

[1] Each value represents the mean of two determinations.
[2] Values followed by the same letter are not significantly different (p≤0.05).

APPENDIX 8a. Analysis of variance of protein solubility at pH 1.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	8143.2760	904.8084	193.01	0.0001
Error	8	37.5029	4.6879		
Corrected Total	17	8180.7789			

R-Square	C.V.	Root MSE	Mean
0.995416	13.35000	2.1651	16.218

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	8142.5439	1017.8180	217.12	0.0001
REP	1	0.7321	0.7321	0.16	0.7030

APPENDIX 8b. Analysis of variance of protein solubility at pH 2.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	2806.8461	311.8718	206.53	0.0001
Error	8	12.0806	1.5101		
Corrected Total	17	2818.9267			

R-Square	C.V.	Root MSE	Mean
0.995714	5.129237	1.2289	23.958

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	2803.5693	350.4462	232.07	0.0001
REP	1	3.2768	3.2768	2.17	0.1790

APPENDIX 8c. Analysis of variance of protein solubility at pH 3.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	2647.0293	294.1144	341.10	0.0001
Error	8	6.8980	0.8623		
Corrected Total	17	2653.9273			

R-Square	C.V.	Root MSE	Mean
0.997401	6.738293	0.9286	13.781

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	2646.3212	330.7902	383.64	0.0001
REP	1	0.7080	0.7080	0.82	0.3913

APPENDIX 8d. Analysis of variance of protein solubility at pH 4.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	78.438100	8.715344	940.36	0.0001
Error	8	0.074144	0.009268		
Corrected Total	17	78.512244			

R-Square	C.V.	Root MSE	Mean
0.999056	9.397361	0.0963	1.0244

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	78.437744	9.804718	1057.90	0.0001
REP	1	0.000356	0.000356	0.04	0.8496

APPENDIX 8e. Analysis of variance of protein solubility at pH 5.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	13.800839	1.533427	8.10	0.0036
Error	8	1.514211	0.189276		
Corrected Total	17	15.315050			

R-Square	C.V.	Root MSE	Mean
0.901129	35.80733	0.4351	1.2150

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	13.571900	1.696488	8.96	0.0028
REP	1	0.228939	0.228939	1.21	0.3034

APPENDIX 8f. Analysis of variance of protein solubility at pH 6.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	5557.4851	617.4983	27.34	0.0001
Error	8	180.6598	22.5825		
Corrected Total	17	5738.1448			

R-Square	C.V.	Root MSE	Mean
0.968516	25.89153	4.7521	18.354

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	5521.8964	690.2370	30.57	0.0001
REP	1	35.5887	35.5887	1.58	0.2448

APPENDIX 8g. Analysis of variance of protein solubility at pH 7.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	8539.4454	948.8273	117.04	0.0001
Error	8	64.8544	8.1068		
Corrected Total	17	8604.2998			

R-Square	C.V.	Root MSE	Mean
0.992463	4.663367	2.8472	61.056

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	8500.6667	1062.5833	131.07	0.0001
REP	1	38.7787	38.7787	4.78	0.0602

APPENDIX 8h. Analysis of variance of protein solubility at pH 8.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	6065.6011	673.9557	63.20	0.0001
Error	8	85.3159	10.6645		
Corrected Total	17	6150.9170			

R-Square	C.V.	Root MSE	Mean
0.986130	4.836770	3.2657	67.517

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	6032.3898	754.0487	70.71	0.0001
REP	1	33.2112	33.2112	3.11	0.1156

APPENDIX 8i. Analysis of variance of protein solubility at pH 9.

Source		DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	9	5854.2882	650.4765	50.12	0.0001
Error		8	103.8206	12.9776		
Corrected Total		17	5958.1088			

R-Square	C.V.	Root MSE	Mean
0.982575	5.294030	3.6024	68.047

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	5828.2961	728.5370	56.14	0.0001
REP	1	25.9920	25.9920	2.00	0.1947

APPENDIX 9. Emulsifying properties of PMM and all acylated concentrates.

Sample	Emulsifying Activity (%)[2]	Emulsion Stability (%)[2]
PMM	39.9 ± 1.1^{g}	68.0 ± 5.4^{c}
3% succinylation	63.2 ± 4.7^{dc}	96.7 ± 1.2^{a}
48% succinylation	69.8 ± 2.2^{c}	86.9 ± 6.5^{b}
53% succinylation	61.4 ± 4.0^{e}	85.5 ± 4.4^{b}
61% succinylation	100 ± 0^{a}	56.5 ± 3.4^{d}
16% acetylation	75.3 ± 0.9^{b}	97.5 ± 0.9^{a}
26% acetylation	53.9 ± 0.8^{f}	96.0 ± 3.4^{a}
42% acetylation	65.9 ± 3.8^{d}	95.5 ± 1.0^{a}
62% acetylation	77.0 ± 1.5^{b}	85.1 ± 1.4^{b}

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents the mean of two determinations.

APPENDIX 10a. Analysis of variance of emulsifying activity.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	8909.9540	809.9958	126.85	0.0001
Error	24	153.2480	6.3853		
Corrected Total	35	9063.2020			

R-Square	C.V.	Root MSE	Mean
0.983091	3.750688	2.5269	67.372

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	8878.2368	1109.7796	173.80	0.0001
REP	3	31.7172	10.5724	1.66	0.2031

APPENDIX 10b. Analysis of variance of emulsifying stability.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	6652.8736	604.8067	45.72	0.0001
Error	24	317.5183	13.2299		
Corrected Total	35	6970.3919			

R-Square	C.V.	Root MSE	Mean
0.954448	4.260086	3.6373	85.381

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	6609.1903	826.1488	62.45	0.0001
REP	3	43.6833	14.5611	1.10	0.3682

APPENDIX 11. Foaming properties of PMM and acylated concentrates.

Sample	Foam Capacity (%)[2]	Foam Stability (%)[2]
PMM	43.3 ± 3.3[g]	74.7 ± 7.6[a]
3% succinylation	53.3 ± 3.3[f]	48.1 ± 4.9[d]
48% succinylation	132.2 ± 1.9[e]	13.4 ± 1.4[d]
53% succinylation	143.3 ± 3.3[d]	10.8 ± 1.2[d]
61% succinylation	157.8 ± 6.9[c]	5.6 ± 1.0[d]
16% acetylation	166.7 ± 0[b]	30.0 ± 2.0[c]
26% acetylation	126.7 ± 5.8[e]	12.3 ± 2.0[d]
42% acetylation	146.7 ± 3.3[d]	12.1 ± 1.1[d]
62% acetylation	188.9 ± 5.1[a]	6.5 ± 2.0[d]

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents a mean of two determinations.

APPENDIX 12a. Analysis of variance of foaming capacity.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	58295.236	5829.524	302.97	0.0001
Error	16	307.860	19.241		
Corrected Total	26	58603.096			

R-Square	C.V.	Root MSE	Mean
0.994747	3.406588	4.3865	128.76

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	58291.940	7286.493	378.69	0.0001
REP	2	3.295	1.648	0.09	0.9183

APPENDIX 12b. Analysis of variance of foaming stability.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	13085.046	1308.505	53.38	0.0001
Error	16	392.186	24.512		
Corrected Total	26	13477.232			

R-Square	C.V.	Root MSE	Mean
0.970900	20.90630	4.9509	23.681

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	13067.494	1633.437	66.64	0.0001
REP	2	17.552	8.776	0.36	0.7045

APPENDIX 13. Water holding capacity of PMM and all acylated concentrates.

Sample	WHC (%)[2]
PMM	157.0 ± 4.2[a]
3% succinylation	0.0[c]
48% succinylation	12.0 ± 2.8[c]
53% succinylation	0.0[c]
61% succinylation	37.0 ± 7.1[b]
16% acetylation	0.0[c]
26% acetylation	0.0[c]
42% acetylation	0.0[c]
62% acetylation	30.0 ± 17.0[b]

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents the mean of two determinations.

APPENDIX 14. Analysis of variance of water holding capacity.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	41799.807	4644.423	120.98	0.0001
Error	8	307.111	38.389		
Corrected Total	17	42106.918			

R-Square	C.V.	Root MSE	WHC Mean
0.992706	23.62434	6.1959	26.227

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	41742.918	5217.865	135.92	0.0001
REP	1	56.889	56.889	1.48	0.2582

APPENDIX 15. Fat absorption capacity of PMM and all acylated concentrates.

Sample	FAC (%)[2]
PMM	190.0 ± 4.7[f]
3% succinylation	208.3 ± 2.4[f]
48% succinylation	235.0 ± 2.4[e]
53% succinylation	360.0 ± 28.3[b]
61% succinylation	401.7 ± 11.8[a]
16% acetylation	270.0 ± 0[d]
26% acetylation	310.0 ± 4.7[c]
42% acetylation	368.3 ± 2.4[b]
62% acetylation	365.0 ± 2.4[b]

[1] Values followed by the same letter are not significantly different ($p \leq 0.05$).
[2] Each value represents a mean of two determinations.

APPENDIX 16. Analysis of variance of fat absorption capacity.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	97278.811	10808.757	104.54	0.0001
Error	8	827.128	103.391		
Corrected Total	17	98105.940			

R-Square	C.V.	Root MSE	FAC Mean
0.991569	3.378989	10.168	300.93

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	97100.345	12137.549	117.39	0.0001
REP	1	178.456	178.416	1.73	0.2254

APPENDIX 17a. Analysis of variance of L.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	700.43926	70.04393	1071.49	0.0001
Error	16	1.04593	0.06537		
Corrected Total	26	701.48519			

R-Square	C.V.	Root MSE	Mean
0.998509	0.409349	0.2557	62.459

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	700.37185	87.54648	1339.24	0.0001
REP	2	0.06741	0.03370	0.52	0.6067

APPENDIX 17b. Analysis of variance of a.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	16.703704	1.670370	263.36	0.0001
Error	16	0.101481	0.006343		
Corrected Total	26	16.805185			

R-Square	C.V.	Root MSE	Mean
0.993961	23.37272	0.0796	0.3407

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	16.698519	2.087315	329.09	0.0001
REP	2	0.005185	0.002593	0.41	0.6712

APPENDIX 17c. Analysis of variance of b.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	21.012593	2.101259	128.58	0.0001
Error	16	0.261481	0.016343		
Corrected Total	26	21.274074			

R-Square	C.V.	Root MSE	Mean
0.987709	0.761613	0.1278	16.785

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	21.007407	2.625926	160.68	0.0001
REP	2	0.005185	0.002593	0.16	0.8546

APPENDIX 18a. Analysis of variance of G'.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	1170893.9	130099.3	54.99	0.0001
Error	8	18928.1	2366.0		
Corrected Total	17	1189822.0			

R-Square	C.V.	Root MSE	Mean
0.984092	13.94205	48.642	348.89

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	1170781.7	146347.7	61.85	0.0001
REP	1	112.2	112.2	0.05	0.8331

APPENDIX 18b. Analysis of variance of G".

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	32407.836	3600.871	29.47	0.0001
Error	8	977.500	122.187		
Corrected Total	17	33385.336			

R-Square	C.V.	Root MSE	Mean
0.970721	22.03327	11.054	50.169

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	32407.833	4050.979	33.15	0.0001
REP	1	0.003	0.003	0.00	0.9964

APPENDIX 18c. Analysis of variance of tan δ.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	9	0.0542136	0.0060237	24.59	0.0001
Error	8	0.0019600	0.0002450		
Corrected Total	17	0.0561736			

R-Square	C.V.	Root MSE	Mean
0.965108	11.00135	0.0157	0.1423

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TRT	8	0.0541011	0.0067626	27.60	0.0001
REP	1	0.0001125	0.0001125	0.46	0.5171