EFFECT OF PROTEIN BINDING WITH PHYTIC ACID ON THE THERMAL GELATION OF BOVINE SERUM ALBUMIN AND CANOLA 12S GLOBULIN AT THE INTERMEDIATE pH RANGE

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Amy Wan-sau Wong

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ABSTRACT

The present study was undertaken to examine how phytic acid interacted with canola 12S globulin at the intermediate pH range and to assess the effect of this interaction on thermal gelation. Bovine serum albumin (BSA) was used to form a model system to study the phytic acid binding to protein and its effect on thermal gelation. The degree of phytic acid binding was determined by using equilibrium dialysis as a function of pH and concentrations of phytic acid and calcium ion. Dynamic rheology was used to assess the influence of the binding on the thermal gelation under the same conditions. The phytic acid binding to BSA and the canola globulin was highly pH-dependent. The binding for both proteins was intensified at pH values below their isoelectric points and the highest binding was always at the lowest pH level regardless of the concentrations of phytic acid and calcium. BSA gels formed at pH 5 were weak and inelastic due to the protein aggregation which was caused by the phytic acid binding and the proximity to the isoelectric point. Although the canola globulin gels formed at pH 5 and 7 (below the isoelectric point) were also weak and inelastic, the role of binding on thermal gelation was insignificant. The presence of calcium only decreased the binding of phytic acid to the canola globulin. Above the isoelectric point, the binding to BSA at pH 5 was moderate but minimal binding was obtained from pH 6 to 9. In the presence of calcium, the binding of phytic acid was discouraged. The rigidity and elasticity of BSA gels formed at this pH range were mainly determined by pH and calcium. The rigidity of the

gels always had the maximum strength at pH 5 and, then decreased from pH 6 to 9. On the other hand, the elasticity of the gels increased as the pH level increased. The effect of phytic acid binding on the thermal gelation at pH 5 was significant, by promoting protein aggregation, but the effect was minor from pH 6 to 9. For the canola 12S globulin, the binding at pH 9 was very low and was influenced by the calcium ion (in 0.01M). Although the most rigid and elastic globulin gels were formed at this pH, the impact of phytic acid binding was small. The mechanism of phytic acid binding was found to be the electrostatic interaction between the negatively charged phytic acid and the positively charged residues on the proteins. This binding occurred at pH values close to and below the isoelectric point. There was no evidence to support the formation of a ternary complex (phytic acid-calcium-protein) at the pH values above the isoelectric point. As a result, only the gel structures formed at pH values below the isoelectric point were influenced by the phytic acid binding.

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I. INTRODUCTION

The gelling ability of protein has a significant role in numerous foods. For example, the gel network can entrap food components, preventing them from leaching while maintaining their distribution in the products. Likewise, thermal gelation of oilseeds and legume proteins can also have major applications in food formulations for the future. However, the presence of phytic acid, which is a common substance in legumes and oilseeds, can influence the gelling ability by complexing with multivalent cations and proteins due to its strongly negatively charged nature. Thus, it may cause problems for utilizing the protein products as gelling agents in food preparations. Although studies focusing on the effects of phytic acid on some of the proteins' functionalities have been done, there was no information for its effect on thermal gelation (de Rham and Jost, 1979; Chen and Morr, 1985; Dev and Mukerjee, 1986; Lapveteläinen et al., 1992).

The overall purpose of the present study was to examine how phytic acid interacted with plant proteins, specifically a canola protein isolate, at the intermediate pH range and to assess the effect of this interaction on thermal gelation. Specific objectives were to:

1. determine the degree of binding of phytic acid to bovine serum albumin (BSA) as a function of pH plus concentrations of phytic acid and calcium ions.

2. determine the thermal gelation properties of BSA under the same

conditions.

3. determine the degree of binding of phytic acid to canola protein as a function of pH plus concentration of phytic acid and calcium ions (values based on results of BSA model).

4. determine the thermal gelation properties of canola protein isolate under the same conditions.

5. investigate the relationship between phytic acid binding and thermal gelation properties for these two proteins.

To successfully incorporate plant protein products into foods as gelling agents, a better understanding of factors affecting the binding behaviour and their effect on the thermal gelation is essential.

II. LITERATURE REVIEW

A. Mechanism for Thermal Gelation Of Globular Proteins

Gels are a form of matter intermediate between a solid and a liquid. They consist of polymeric molecules cross-linked to form a tangled, inter-connected molecular network immersed in a liquid medium (Oakenfull, 1987). Continuous networks with a certain degree of order are most often exploited in food products. Food protein gels are used in the food industry in a wide range of products, both traditional and novel, and this use is increasing rapidly. Plant storage proteins not only have nutritional value but also provide functional properties in food systems. Among all these functionalities, gel-forming ability is one of the major properties of plant proteins in food products. Recently, many products made with plant proteins have been successfully formulated and marketed, such as soy sausage and soy hamburger.

Most plant storage proteins share a common characteristic - the globular shape of the molecule. To understand the properties of the protein gels, the molecular structure of the protein, the inter-/intra-molecular forces that give protein stability and the formation of junction zones have to be carefully examined (Oakenfull, 1987). Gelation occurs when the molecules unfold (or partly unfold) and then refold (or partly refold) in different conformations so as to form a network. There are mainly two kinds of gelling mechanism: thermally and chemically induced gelations (Clark and Lee-Tuffnell, 1986). In the case of thermal gelation, heat energy is the inducing force to open up the protein

molecules.

Formerly, a two stage process for thermal gelation of the globular proteins had been proposed (Ferry, 1948). In the first stage, native protein molecules were denatured by heat and unfolded into long polypeptide chains. Subsequently, there was association between the polypeptides, and eventually a continuous network was formed. The extent of the association depended on a balance of attractive and repulsive forces between the polypeptide chains under highly specific conditions. Lately, there was more and more evidence suggesting that the protein molecules did not unfold into polypeptide chains but, instead, the molecules partially unfolded and still retained the globular shape (Nakamura et al., 1984; Clark and Tunffnell, 1986; Oakenfull, 1987). A intermediate, soluble aggregate, was formed by positioning the partially unfolded molecules on one another. Thus, it looked like a strand of beads. The balance of attractive and repulsive forces again determined the extent of the aggregation and the orientation of the "beads". This mechanism has been referred to as a corpuscular structure formation. The calculated protein requirement for this type of formation would certainly be greater than the requirement for chain interactions. In most cases, a 7 to 10 % protein solution was required for any adequate structural formation (Hegg, 1982; Arntfield, 1989). The new scheme for thermal gelation of globular proteins is as follows (Arntfield, 1989):

native --> partially unfolds --> soluble aggregate --> network

Thermal gelation occurred when a protein solution was heated above the denaturation temperature of that particular protein. Protein denaturation was the chief and determinant factor in gelation. It should preceed the association of protein molecules and

increase the potential for interaction among the molecules (Clark and Lee-Tuffnell, 1986; Arntfield, 1989; Matsudomi et al., 1991). In addition, by increasing the difference between aggregation (T_A) and denaturation (T_D) temperatures, gel structure could be improved (Hegg et al., 1978, 1979). However, the delayed structure development in relation to the T_D value did not necessarily result in good network formation as the presence of various anions could improve network formation (Arntfield et al., 1989).

As mentioned, gels formed only under highly specific conditions; the balance of attractive and repulsive forces between the protein molecules determined which type of network structure would form (opaque or transparent). The balance of forces depended on the gelling conditions, such as the concentration of protein, heating temperature, time of heating, pH values, ionic strength and protein binding activity with other substances. In general, gels were only formed in the conditions that represented the boundary between protein aggregation and solubility (Hegg, 1982; Arntfield, 1989). The boundary for any globular protein could be predicted by knowing the titration curve, amount of salt present and some physical data, such as the isoelectric point and pH-induced transition. On the other hand, no common simple physical characteristic of globular proteins which was crucial for gel formation could be identified, although high contents of disulphide bridges, sulphydryl groups and intramolecular β-sheet structure in the native state had been shown to facilitate gel network formation (Hegg, 1982). The molecular weight had recently been shown to influence the hardness and the gel strength (Wang and Damodaran, 1990). It was indicated that the hardness or gel strength of typical globular protein gels was fundamentally related to the size and shape of the polypeptides in the gel network rather

than to their chemical nature such as the amino acid composition and distribution. It was also shown that the globular proteins having a weight-average molecular weight less than 23,000 could not form a self-supporting gel network at any reasonable concentration (Wang and Damodaran, 1990). Moreover, the intermolecular hydrogen bonding between segments of β-sheets oriented either in parallel or in antiparallel configurations may serve as junction zones in the gel network (Wang and Damodaran, 1991). The conformation of protein molecules, and resulting surface properties, as well as the influence of conformational changes associated with different environmental conditions, affect the interactions between proteins. These effectively altered the attractive and repulsive forces necessary for network formation (Arntfield, 1989). The intermolecular interactions, including electrostatic and hydrophobic interactions, hydrogen bonds and disulfide bonds, were the main attractive contribution in the balance of the forces. Theoretically, the types of networks that formed with charge manipulation could be used as a model for the networks that result from the manipulation of other attractive forces since the electrostatic charge appeared to provide the principle repulsive force. (Arntfield, 1989).

B. Phytic Acid

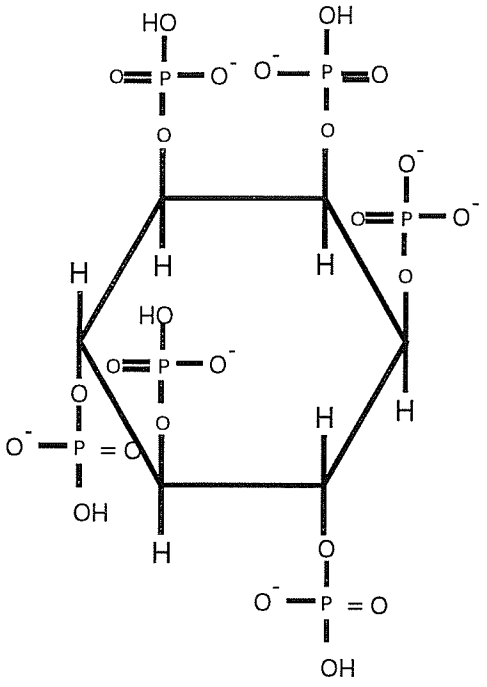
Phytic acid, a 6-carbon ring consisting of six phosphates, is one of the polyphosphorylated inositols commonly found in nature. It has been found in the greatest amounts in cereal, legumes and nuts. In general, phytate constitutes about 1 to 2% by weight of many cereals and oilseeds. The term phytin refers to a calcium-magnesium salt of phytic acid; and phytate means the mono to dodeca anion of phytic acid. In order to

comprehend the interaction between phytic acid and protein, and its effect on thermal gelation, a full understanding of the properties of phytic acid is necessary.

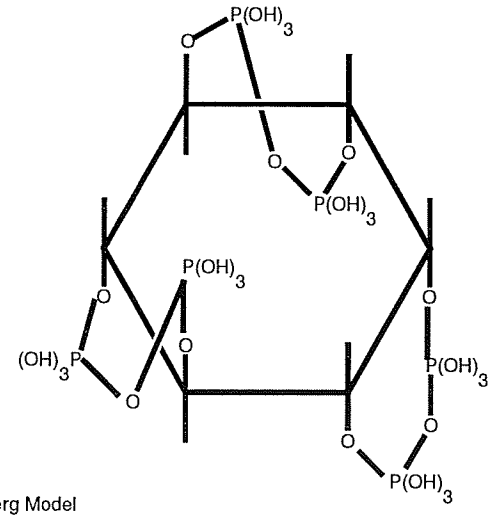
1. Structure and Chemistry

A argumentative issue has been developed over the detailed type of structure of phytic acid in the literature. This conflict involved the conformation as well as the configuration of the molecule. Its chemical structure was primarily questioned on the basis of multiple isomeric forms of hydroxyl groups. To uncover the precise structure of phytic acid is unquestionably crucial, since the mechanism for any interaction of phytic acid shall be explained by its structure and chemistry. In fact, phytic acid does react with many other food components and these interactions are responsible for its adverse nutritional effect in high-phytate diets and for the impact on protein usage in food formulation.

Two molecular models - the Anderson and Neuberg structures have been proposed as being the correct conformation of phytic acid. They are shown in Fig. 1 (Cheryan, 1980). The Anderson structure, given by the formula $C_6H_{18}O_{24}P_6$, is a symmetrical hexaorthophosphate while the Neuberg structure, given by the formula $C_6H_{24}O_{27}P_6$, is asymmetrical. The Neuberg structure, as shown in Fig. 1b, can be distinguished by having three P-O-P linkage between pairs of adjacent phosphates. Since the two structures only differ by three water molecules, it is also tempting to conclude that they may exist simultaneously in equilibrium with each other (Brown et al., 1961; Erdman, 1979). In addition, the Anderson structure can alternatively be described as the



a) Anderson Model



b) Neuberg Model

Figure 1. Two Possible Molecular Structures of Phytic Acid
a) Anderson structure b) Neuberg structure (Cheryan, 1980)

degradation product of the Neuberg structure.

A large number of studies supported the Anderson structure based on pH-titration and conductivity measurements, chemical hydrolysis, nuclear magnetic resonance, X-ray crystallography plus proton-NMR techniques (Cheryan, 1980; Maga, 1982). Likewise, a number of workers using a variety of techniques also provided convincing evidence to support the Neuberg structure (Cheryan, 1980; Alli and Baker, 1981; Maga, 1982). Nevertheless, current literature appears to favour the Anderson structure simply because many of the physicochemical properties, interactions, and nutritional effects can be better explained in terms of the Anderson model. Thus, it is now generally accepted that the structure proposed by Anderson as shown in Fig. 1a is probably the correct one (Cheryan, 1980). Besides that, there is a disagreement over the configurational positions of the phosphates among the proponents of the Anderson structure (Johnson and Tate,1969; Blank et al., 1971). Some researches have suggested that the phosphate on carbon-2 was on the axial plane while all other phosphates were on the equatorial plane; whereas some others have reached a totally opposite conclusion. The two isomers are shown in Fig. 2 (Johnson and Tate, 1969; Blank et al., 1971). All the conflicting conclusions about the precise structure of phytic acid may due to the nature of extracting material and the uncertainty in the assay procedure (Cheryan, 1980; Maga, 1982). This is because phytic acid is thought to be unstable. It has different crystalline forms depending on the degree of hydration, and also various configurational changes at different pH levels. It seems that the controversy of the molecular structure of phytic acid will continue.

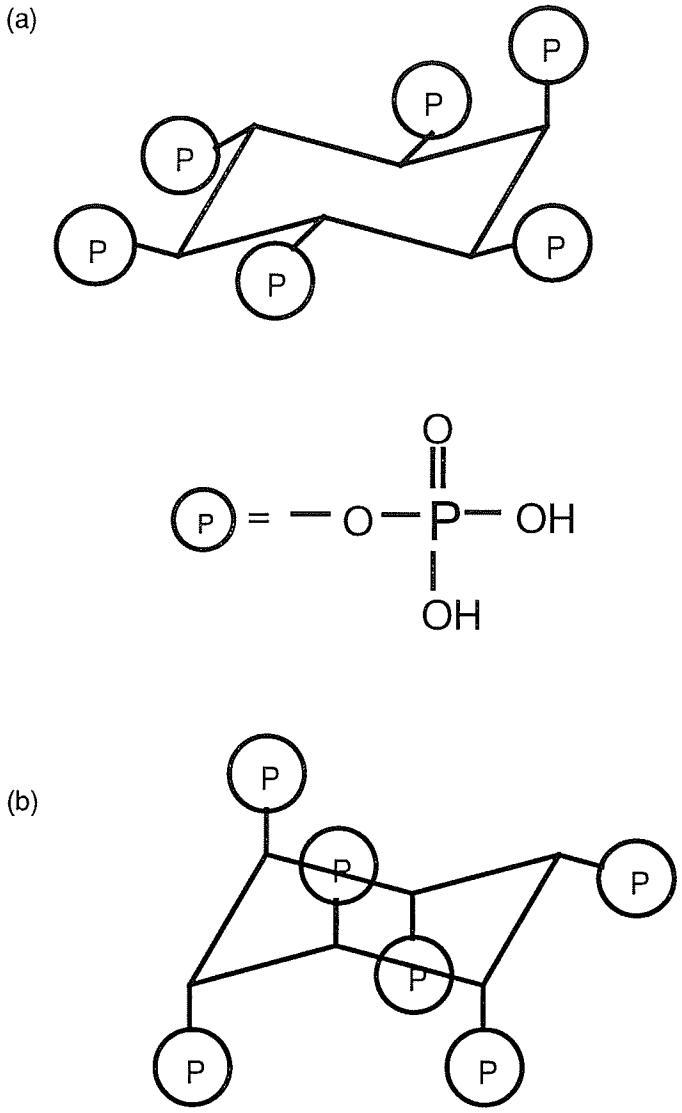


Figure 2. Two Possible Configurational Isomers of the Anderson Structure (Johnson and Tate, 1969; Blank et al., 1971)

a) phosphate on C-2 is in axial plane b) phosphate on C-2 is on equatorial plane

In the following discussion, the Anderson structure is adopted to explain the chemistry and mechanisms of phytic acid interactions.

On the basis of the Anderson structure, the proper scientific name for phytic acid is myoinositol 1, 2, 3, 4, 5, 6-hexakis-dihydrogen phosphate (IUPAC-IUB, 1968). Using the Henderson-Hasselbalch equation to determine the ionizable protons of phytic acid, 6 protons are found strongly dissociated with a pK_a of about of 1.8, 2 are weak acid functions with a pK_a of 6.3, and 4 are feebly dissociated with a pK_a of 9.7 (Cheryan, 1980). Similar results are obtained by using proton NMR-pH titration methods: 6 in the strong acid range (pK_a of 1.5), 1 in the weak acid range (pK_a of 5.7), 2 others in the middle range (pK_a of 6.8-7.6), and 3 in the extremely weak acid range (pK_a >10). In both cases, there are 12 replaceable protons in the phytic acid molecule. At pH values (~pH 6.0) normally encountered in foods, phytic acid is strongly negatively charged as indicated in Fig. 1a and is very reactive with other positively charged groups such as metal ions. Due to its multiplicity of reactive phosphate groups, phytic acid can complex a cation within a phosphate group itself, between two phosphate groups of a molecule, or between phosphate groups of different phytic acid molecules (Cheryan, 1980).

2. Interaction of Phytic Acid with Metal Ions

Understanding the nature of the interaction between phytic acid and metal ions is very important since multivalent metal ions can act as bridges for other negatively charged substances to bind with phytic acid.

Phytic acid can form stable complexes with metal ions, and a variety of structures

are possible for these complexes. In Fig. 3, four different theoretical structures have been proposed (Nolan et al., 1987). A multivalent metal ion can form more than one bond within one phosphate group as in structure I. In structures II and III, two or more phosphate groups from the same or from different phytate ions will complex to one metal cation. Sometimes, two metal ions can bind to single phosphate group giving structure IV. Unfortunately, the structural information on these complexes is limited.

Most salts of phytic acid (or phytates) in plant materials, such as sodium or potassium phytate, are normally relatively soluble and can be washed away by water. The formation of "insoluble" phytates, calcium/magnesium salts, usually occurs as a result of heat treatment and/or changes in pH and ionic strength (Cheryan, 1980).

Solubility studies were often used to investigate the interaction of phytic acid with metal ions since precipitation of phytates could be used as an indication of binding. The solubility and stability of various metal-phytate complexes were determined by measuring a drop in pH (Cheryan, 1980). The displacement of acidic protons by metal ions and the shift of the phytate ionization equilibrium cause the pH to drop. The magnitude of the pH drop indicates the complexing tendency and is a qualitative measure of stability. Based on the pH-drop method, zinc formed the most stable complexes with phytic acid, followed by copper, nickel, cobalt, manganese, calcium, and iron, in decreasing order of stability. However, the complex behavior of phytic acid with metal ions is more complicated. The behavior cannot only be affected by other co-exiting metal ions and chemical substances but also by the presence of proteins (Cheryan, 1980; Nolan et al., 1987; Gifford-Steffen and Clydesdale, 1993). The amounts of metal ions,

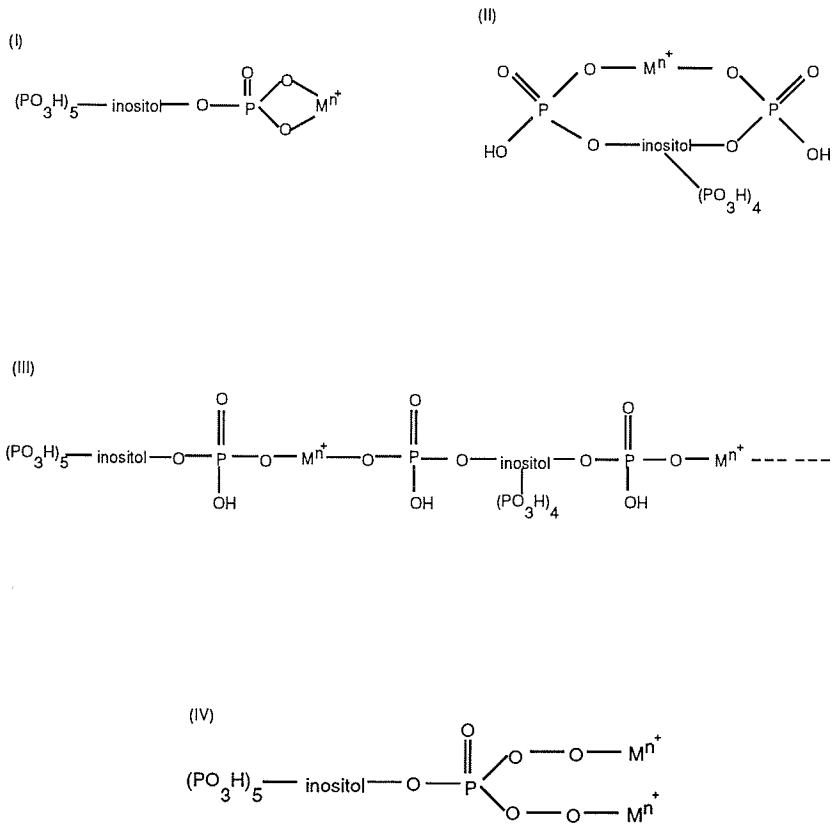


Figure 3. Four Theoretical Structures of Metal Phytate
(Nolan et al., 1987)

I) reaction with single phosphate group. II) with two phosphate groups in same molecule. III) with phosphate groups from different phytate ions. IV) two metal ions bind to single phosphate group.

pH and the ionic strength also play significant roles.

In the absence of protein, the solubility of some common metal-phytate complexes in aqueous solution were compared at various pH levels (Cheryan, 1980). The calcium (Ca) and magnesium (Mg) salts of phytic acid are soluble at low pH values. Between pH 5.5 and 6.0, there is a rapid decline in calcium phytate solubility, while the magnesium phytate solubility decreases between pH 7.2 and 8.0. The precipitation of phytates results in tri-, tetra-, penta- and hexa-metal complexes. The mono- and di-metal complexes are the only species soluble in water (de Rham and Jost, 1979). The formation of these different forms of metal complexes has been shown to depend on the metal to phytic acid ratio (Nolan et al., 1987). For example, at [metal]/[phytic acid] ratio larger than 5, the main species of complexes is $metal_5$(phytate) at a given pH. The extent of binding depends on the pH level: generally the higher the pH greater the extent of binding. Most metal ions (ie. Mg^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+} and Zn^{2+}) have the similar complexing behaviours.

The solubility behaviour of mixed salts of phytic acid is also complicated. Interaction between zinc and calcium in solutions of sodium phytate was studied most (Cheryan, 1980). At high calcium, low zinc concentrations, insoluble calcium-phytate-zinc complexes were formed. At high concentrations of zinc and calcium, calcium competed for positions on the phytate molecule which reduced the amount of zinc that precipitated. In other studies, the level of phytic acid were suggested to be equally critical (Graf and Eaton, 1984; Champagne, 1987). Calcium ions could potentiate zinc ion precipitation at high phytic acid:zinc ratios. At low phytic acid:zinc ratios, calcium competed with zinc for binding sites. At higher concentrations of calcium ions, there was

more potentiation or competition. In the case of magnesium, it formed a mixed precipitate with calcium (Graf, 1986). Low concentrations of magnesium or calcium did not precipitate phytate, but if either were increased, a point was reached at which both precipitated and hence the solubility of one was inversely proportional to the other. Solubility studies on mixed salt systems are sorely lacking in the literature although real foods are mixed systems of varying ionic strengths.

The presence of other chemical compounds, particularly strong competitive chelators, also affects the solubility of mineral phytate (Cheryan, 1980). Ethylene diamine-tetra-acetic acid (EDTA) at alkaline pH values binds to cations preferentially, and thus inhibits the formation of mineral phytate. Certain amino acids are able to inhibit the formation of mineral phytates presumably by the same mechanism.

In soybean, rapeseed, cottonseed and peanut protein systems, the solubility of phytic acid somewhat parallels the solubility behavior of the proteins (Cheryan, 1980). This solubility profile of phytates is quite different from the cases in the absence of protein. These observations have been used to suggest the possibility of interactions between phytic acid and protein.

When both zinc and iron were added to a wheat bran fraction, a significant decrease in the solubility of phytic acid, phosphorus, protein and endogenous calcium resulted (Platt and Clydesdale, 1987). If they were added to the sodium phytate alone, no precipitate formed. This indicated that complexation only occurred when protein and/or endogenous calcium, a multivalent cation, was present. The interactions among protein, phytate, zinc, and calcium at varying millimolar ratios of phytate x calcium:zinc

were carefully studied in soy protein concentrate (Gifford-Steffen and Clydesdale, 1993). At low pH values (pH 2.2), as the concentration of calcium ion was increased in the multi-ratio studies, the soluble protein and phytic acid both increased significantly whereas the soluble calcium ion concentration was lower. It was probable that as the calcium concentration increased, the protein and phytic acid interacted to a lesser extent and formed less insoluble complex due to the interference from calcium ions. Interestingly, the addition of Zn had no significant effect on the concentrations of soluble protein, calcium and phytic acid at this low pH. At higher pH values (pH 5.5), all the soluble calcium ion, zinc ion and phytic acid decreased significantly as the concentration of calcium ion increased. It appeared that increased repulsion occurred between both the negatively charged phytic acid and protein which increased their solubilities. Thus, the interactions among zinc ions, calcium ions and phytic acid were enhanced resulting in the formation of calcium phytate and calcium-zinc phytate. Increasing zinc concentration under similar conditions gave similar results.

3. Interaction between Phytic Acid and Protein

a. Low pH. Under acidic conditions, which are below the isoelectric point of proteins, the precipitation of proteins by phytic acid was regarded as the formation of an insoluble unionized salt since the protein was positively charged (Cheryan, 1980; Prattley et al., 1982). Proteins, due to their basic amino acid content, are positively charged under acidic conditions. In some cases, these basic residues can retain their positive charge up to pH 9. According to the following equation, the protein acts as the cation

and the acid is the anion.

$$Protein^{n+} + n\ Acid^{-} \text{ --------> } Protein\text{-}Acid_n$$

Thus, the protein-phytic acid interaction at pH values lower than the isoelectric point is a result of strong electrostatic interactions. The possible structure of phytic acid-protein complex at low pH is shown in Fig. 4 (Cheryan, 1980).

In many studies, using different protein sources, the formation of unionized soluble protein-phytic acid complexes at low pH was evidenced (Cheryan, 1980). In the studies with human serum albumin, a molar binding ratio of 86 with an association constant of about 10^6 at pH 4.1 was reported (Barré and Van Huot, 1965). The ratio was associated with the sum of the terminal amino groups and lysine residues. An additional set of 23 sites with lower binding affinity were correlated with histidine content. A further additional 35 sites became available only at pH 2.45 or lower. A follow-up study was done on ovalbumin. An association constant of $5x10^4$ and 80 binding sites were obtained. This finding accounted for the sum of lysine and arginine residues but the histidines in ovalbumin somehow were masked for the interaction with phytic acid. The binding mechanism of phytic acid with bovine serum albumin was also studied (Prattley et al., 1982). In the absence of calcium, the binding extent peaked at pH 3 and steadily declined as the pH increased (up to 12.0). At pH values below 4.0, a thick white precipitate was observed in samples. The decline in binding could be attributed to the repulsion of the phytic acid molecules by the increasingly negatively charged protein. At pH 3.0, the albumin was found to have 78 binding sites with a binding constant of 2.3 x 10^5. However, the 78 determined binding sites, did not match the total available amino

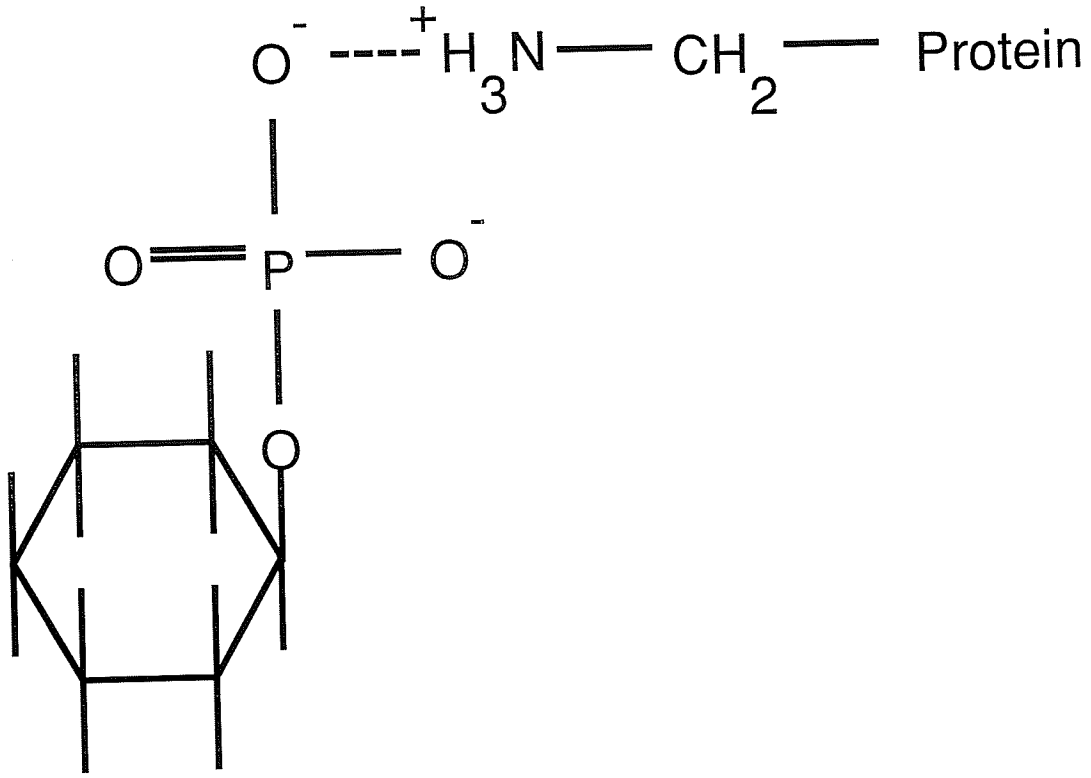


Figure 4. The Structure of Phytic Acid-Protein Complex at Low pH (Cheryan, 1980)

acid content of bovine serum albumin: lysine, 58; histidine,16 and arginine, 19 (total of 93). In the presence of calcium (2.0%), there was a reduction in bound phytic acid and dissolution of the precipitate at low pH. This was probably the result of competition between protein and calcium for binding sites on the phytic acid. The low pH complex was not disrupted even at high temperatures of about 100°C.

The soy protein system is the most extensively studied system for phytic acid interactions. The pH region for binding between the soy glycinin and phytic acid was studied by using a gel filtration technique (Cheryan, 1980). Binding activities only occurred at pH values below 4.9. The extent of binding was found to increase with decreasing pH, from a value of zero at the isoelectric point to a maximum of 424 equivalents of phytate per mole of glycinin dimer at pH 2.5. The equivalent value correlated well with the total number of cationic groups of glycinin at pH 2.5, including lysines, histidines, arginines as well as 12 amino-terminal groups of the glycinin dimer. At pH 3.0, the two major classes of binding sites were the sites that were freely accessible on the protein surface and at the carboxylate groups which are involved in ionic interactions. The former class accounted for more then 75% of the total binding sites. Similar results had been obtained in later works (Grynspan and Cheryan, 1989).

This strong phytic acid-protein interaction at acidic pH values is the reason why protein isolates, prepared by isoelectric precipitation, often contain high levels of (i.e. as much as 60 - 70% of original) phytic acid.

b. Intermediate pH. In this pH region, the salts of phytic acid are somewhat soluble. There was considerable evidence indicating the likelihood of phytate-protein

interaction (Cheryan, 1980; Martens, 1982). It might be due to the conformational changes to the protein with increasing pH that allowed localized binding of small molecules to positively-charged basic amino acid residues (Prattley et al.,1982). Many investigations, however, found that increasing the concentration of multivalent cations (i.e. calcium, and zinc) could enhance the integrity of the protein-phytate complex in soy proteins' system (Cheryan, 1980; Parttley et al., 1982; Nosworthy and Caldwell, 1988). Furthermore, many molecules of calcium and phytic acid were found to bind to a single soy protein molecule (Saio et al., 1968). Thus, the behaviour of the phytate-protein interaction in this region appeared to be strongly influenced by the formation of a salt linkage or an alkaline-earth ion bridge. The following mechanism was proposed for the state of the phytate-protein complex at the intermediate pH region (Cheryan,1980):

Protein + Cation + Phytic acid ⇆ (Protein-Cation-Phytic acid)

However, the direct binding of phytic acid with proteins is possible, as mentioned above, with a few specific terminal amino groups and epsilon amino groups of lysine. Since these groups are still protonated at pH below 10, it is unlikely to take place (Cheryan, 1980). As a result, the formation of a ternary complex is suggested to be the dominant reaction (Grynspan and Cheryan, 1989).

Analysis of binding data has indicated that the most probable binding sites of multivalent cations on a protein are the imidazole groups of histidine (Cheryan, 1980). This is because chelation of metals by histidine is much stronger than that of other amino acids. Neutral nitrogen and negative oxygen groups have the same affinities for both protons and metal ions. The binding activities can only occur with unprotonated

imidazole groups of histidine. Since its pK_a value in general is 6 to 8, it is not surprising that some studies have reported no binding activity in this pH range. The possible structure of the phytate-protein ternary complex is shown in Fig. 5 (Cheryan, 1980).

The strength of the protein-cation-phytate linkages increases with increasing pH (up to ~ pH 10) because proteins become more negatively charged. Several methods were used to dissociate such ternary complexes (Cheryan, 1980). Firstly, it was done by reducing the concentrations of cations or free phytic acid in the reaction system. The removal of reactants caused the reaction to shift to the left, resulting in a dissociation of the ternary complex. Interestingly, the increased concentration of calcium ions could also destabilize the ternary complex and precipitate phytic acid as calcium phytate at the neutral intestinal pH (Graf, 1986). Another method for dissociating the complex was by adding EDTA to the system because cations preferentially bound to EDTA instead of phytic acid. Finally, the addition of more than 8.5% NaCl to the system could also dissociate the complex by disrupting the salt bridges. This resulted in a precipitation of phytic acid. The possible explanation for this dissociation was that the addition of excess sodium ions replaced the cation and phytic acid together, and the Na^+ ion itself formed a complex with the protein. Later work had again confirmed that the dissociation of protein along with the precipitation of calcium phytate was dictated by the Ca:phytic acid ratio and the sodium ion concentration (Grynspan and Cheryan, 1989). High temperature also had a disruptive effect on the ternary complex (Prattley et al., 1982).

From the isoelectric point of protein to the pH at which the imidazole groups of histidine are protonated (in general is pH 6.0 to 9.0), the mechanism of the protein-phytic

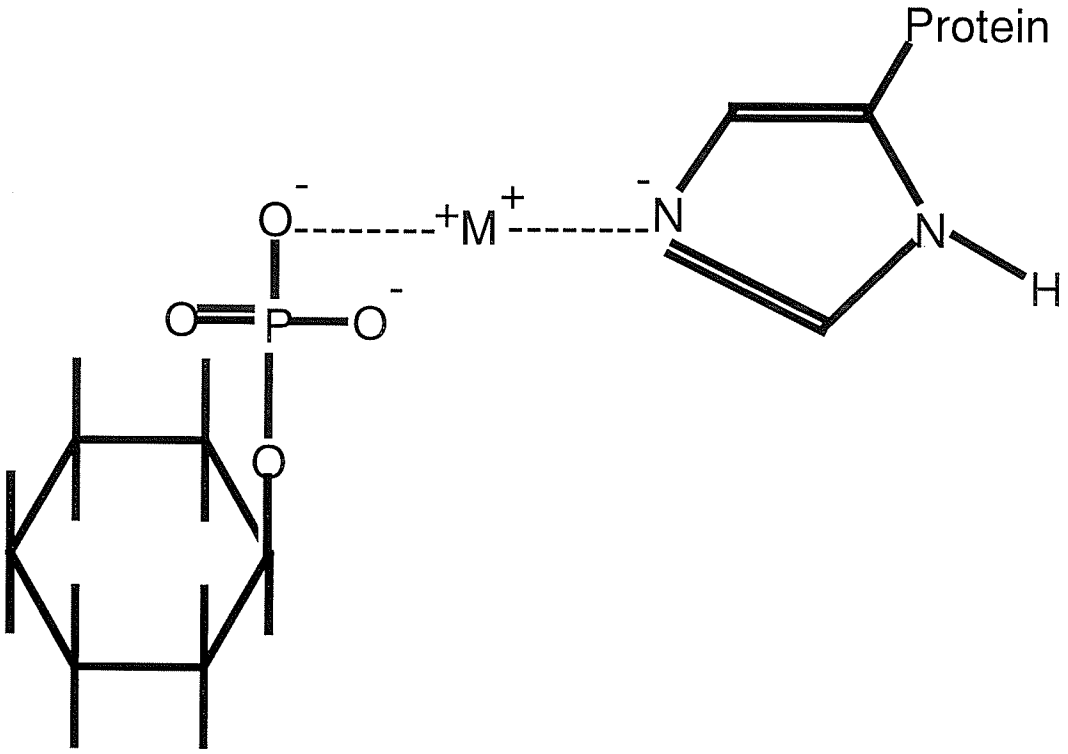


Figure 5. The Structure of Phytate-Protein-Metal Ternary Complex (Cheryan, 1980)

acid interaction is somewhat uncertain (Cheryan, 1980). As mentioned before, many early investigations had failed to determine the interaction in this pH region. Proteins in this intermediate pH have net negative charges; and, also phytic acid is negatively charged. Nevertheless, binding of phytic acid to the negatively charged protein was observed (Prattley et al., 1982; Grynspan and Cheryan, 1989).

c. Extreme high pH. Under extremely basic conditions, (pH 10 or above) phytic acid in soy protein extracts became markedly insoluble and came out of solution as insoluble salt. A similar observation was reported for the rapeseed protein system. It showed that there was a sharp decrease in phytate solubility above pH 10 (Cheryan, 1980). The ternary complex, which was formed at the lower pH region, was thought to dissociate with the formation of insoluble cation-phytic acid complexes and soluble Na proteinate in the presence of excess sodium ions at a pH above 10. The mechanism is shown in the following equation (Cheryan, 1980; Prattley et al., 1982).

$$\text{Protein-Cation-PA}_{(s)} + \text{Na}^{+}_{(aq)} \leftrightarrows \text{protein-Na}_{(aq)} + \text{Cation-PA}_{(s)}$$

The phenomenon might be due to the shift to the right of the equation as the Na^+ ion concentration increased with the addition of alkali. Certain minimum concentrations of multivalent cations and also sodium ions were necessary for the phytate to precipitate out. Phytic-free rapeseed protein could be isolated by extracting the dehulled and partially defatted meal at pH 11.1 and then precipitating at pH 5.1 in the presence of carboxymethylcellulose (Atwal et al., 1980).

In summary, phytic acid does interact with proteins depending on the pH. However, the nature of the interaction is not completely understood. Studies at the

intermediate and extreme pH regions are often difficult to interpret. Moreover, abnormal Scatchard plots, used to plot binding behavior, are usually obtained. Some researchers have concluded that the ability of food proteins to bind phytic acid or minerals appears to be related to its source. In addition, the amino acid composition is not the key component in binding, but it is the accessibility of the binding sites that determines the binding (Gifford and Clydesdale, 1990). In this study, special attention is given to the interactions at the intermediate pH range since it includes the pH values of most foods.

C. Canola Proteins

Rapeseed/canola is a member of the *Brassica* family. Summer rape (*B. napus*) and turnip rape (*B. campestris*) are the cultivars grown in Canada (Sosulski, 1975). The name "canola" was adopted by the rapeseed industry in Canada since 1978 to identify the new cultivars low in both erucic acid and glucosinolates (McCurdy, 1990). In January 1985, the U.S. Food and Drug Administration granted GRAS (Generally Recognized As Safe) status to its oil (McCurdy, 1990). It is now a major oilseed in Canada, Europe and Japan. Worldwide, it currently ranks fourth after soybean, palm and sunflower. Although the commercial defatted meal contains about 44% protein, it is only utilized as livestock feed supplement due to the presence of antinutrients (glucosinolate, phenolics, phytate and fibre). The commercially defatted meals from the canola oil industry are not currently used for producing any protein materials for human consumption.

Today, production of canola protein isolates is done only at an experimental scale. Alkali extraction-acid precipitation is the traditional procedure for isolation of storage

proteins (McCurdy, 1990). It has been employed with canola to isolate proteins from meal or flour. The protein content of canola protein isolates is about 90% or higher. Protein extraction can also be accomplished by using dilute acid, sodium chloride, and/or sodium hexametaphosphate; and, protein can be recovered not only by isoelectric precipitation, but also by heat, acidic polymers and ultrafiltration (McCurdy, 1990). Additional steps are often required for removing antinutrients. For example, glucosinolates can be reduced by activated carbon, acylation and dialysis. The reduction of phenolic compounds is done by alcohol washing. Acylation, dialysis and microbiological fermentation are used to remove phytic acid (McCurdy, 1990; Nari and Duvnjak, 1990). Recently, a process involving a mild salt extraction and precipitation by water dilution was adapted for use with canola (Ismond and Welsh, 1992). Using this process, the protein isolate contained very low residual levels of antinutritional substances. The levels of phytic acid, glucosinolates and phenolic compounds were reduced to 24, 7 and 15% of the raw meal, respectively (Welsh, 1988). No major changes in protein conformation were reported with this procedure.

1. Properties of Canola Protein

There is a wide range of nitrogen-containing compounds in canola; only 80% of these compounds are true proteins. Others are free amino acids, nucleic acids, glucosinolates and hull nitrogen (Sosulski, 1975). The true proteins consists of different fractions, namely the salt-soluble globulins, water-soluble albumins and alkali-soluble proteins (Norton, 1989). The two major protein fractions, which account for about 60%

and 20% of the total proteins respectively, are the neutral high molecular weight (about 300,000±10,000 g/mol) 12S fraction and the basic (molecular weight of 13,800 g/mol) 1.7S fraction (Schwenke et al., 1983; Ericson et al., 1986). Since the usefulness of protein materials in food applications is principally determined by the nature and the characteristics of the protein, it is crucial to characterize all the protein fractions present in rapeseed/canola.

a. 1.7S protein fraction. The 1.7S protein, or napin, is very basic with an isoelectric point of approximately 11.0 and represents an albumin which does not precipitate at its isoelectric point (Lönnerdal and Janson, 1972). The protein molecule has a molecular weight between 12,000 and 14,000 g/mol. It is composed of two disulfide-linked polypeptide chains with molecular weights of 9,000 and 4,000 g/mol (Ericson et al., 1986). The long and the short polypeptide chains consist of 86 and 29 amino residues respectively; and, the protein is rich in glutamic acid. Based on the amino acid analysis, almost all of the glutamic and aspartic acids are in the amide form giving the protein its basic characteristic (Lönnerdal and Janson, 1972). Proline and alanine are found to be the N-terminal groups for the long and the short chains respectively. For both peptide chains, only glycine is identified as the C-terminal residue. In addition, there are five cysteine groups found in the long chain and two in the short chain. Thereby, two disulphide bridges can be found between the peptide chains and one intrachain disulphide bridge is present in the long chain The composition of amino acid of 1.7S fraction is shown in Table 1 (Ericson et al., 1986).

Table 1: Amino Acid Analyses (gAA/100g) of Napin (1.7S) Chains (Ericson et al., 1986)

Amino Acid	Heavy Chain	Light Chain
Lysine	4.67	3.01
Histidine	1.93	1.75
Arginine	2.85	1.62
CM-Cysteine	5.88	2.02
Aspartic acid	2.22	---
Threonine	3.90	---
Serine	3.65	2.29
Glutamic acid	20.44	7.28
Proline	9.76	2.04
Glycine	4.63	2.10
Alanine	3.05	2.58
Valine	6.00	---
Methionine	1.82	1.05
Isoleucine	2.69	1.00
Leucine	6.25	1.91
Tyrosine	1.05	---
Phenylalanine	1.00	1.00

b. 12S protein fraction. The 12S protein has a well balanced amino acid composition and high protein efficiency ratio (Sosulski, 1975; Ohlson, 1985; McCurdy, 1990). The essential amino acid pattern is comparable to or even better than the FAO reference protein (Liu et al.,1982). It is high in glutamic acid and aspartic acid, with considerable amounts of arginine but low amounts of sulfur containing amino acids such as cysteine and methionine. In Table 2, the amino acid composition of 12S globulin isolated by salt extraction and dilution is shown (Burgess, 1991).

Table 2: Amino Acid Composition of the 12S Canola Globulin (Burgess, 1991)

Amino Acid	12S (gAA/100g protein)
Aspartic acid	9.59
Glutamic acid	24.41
Serine	4.59
Glycine	4.43
Histidine	2.51
Arginine	7.27
Threonine	3.94
Alanine	3.72
Proline	3.72
Tyrosine	3.08
Valine	7.68
Methionine	1.33
Cysteine	0.25
Isoleucine	4.77
Leucine	7.35
Phenylalanine	5.28
Lysine	3.77
Tryptophan	2.32

There are four different types of polypeptide chains in the 12S globulin with molar masses of 18,500, 21,100, 26,800 and 31,200 g/mol (Schwenke et al., 1983). Two of these polypeptide chains form a monomeric subunit with molar mass of approximately 50,000g/mol (2-3S). Of these two chains, the smaller one is a basic polypeptide chain, and the larger one is an acidic polypeptide chain (Derhyshire et al., 1976). The two polypeptides are joined by a disulfide bond (Schwenke et al., 1983). For each 12S

protein molecule, there are six of these subunits. Therefore, the 12S globulin has a molar mass of 300,000g/mol. This protein contains 13 intramolecular disulphide bridges and 5 thiol groups, also inside the molecule (Schwenke et al., 1981). These subunits are arranged in the form of a trigonal antiprism with dihedral point group symmetry of 32 (Reichelt et al., 1980). The molecule has also been found to have a morula structure with an almost spherical shape and a maximum diameter of 11.2nm (Mieth et al., 1983a; Schwenke et al., 1983). In addition, the globulin consists of 11%, 31% and 58% for α-helical, β-sheet and aperiodic secondary structures, respectively (Schwenke et al., 1983).

In the presence of urea, at differing ionic strengths or varying pH levels, the globulin molecule dissociated into a trimeric 7S unit when the ionic strength was 0.5 or lower (MacKenzie, 1975). However, it was reversible by increasing the ionic strength (Schwenke et al., 1983). Further dissociation into 2-3S monomeric subunits would occur in the presence of 6M urea, especially in an acidic pH of ≤ 3.6 (Goding et al., 1970). In this case, the dissociation was irreversible. The 2-3S monomer could again dissociate into acidic and basic domains in the presence of a disulphide bond breakers, such as mercaptoethanol (Schwenke et al., 1983; Mieth et al., 1983b). The dissociation-association profile revealed that the association of monomers was not stabilized by covalent disulphide bonds, but by the noncovalent interactions, especially entropically driven hydrophobic interactions (Prakash and Rao, 1986).

The 12S canola globulin was found to be relatively hydrophobic when compared to other oilseeds (Schwenke et al., 1981). Based on the amino acid composition of the globulin, the estimated average hydrophobicity value and nonpolar side chain frequency

were the highest but the polarity was the lowest among oilseeds.

Using isoelectric focusing in urea (6M), the isoelectric point of 12S globulin was determined to be in the pH range of 6.0 - 7.3 (Quinn and Jones, 1976). When determined by an LKB column in a sucrose density gradient (pH 3.5 to 10.0) at 4°C, it was found at pH 7.25±0.10 (Schwenke et al., 1981). The discrepancy might be due to the protein interaction with reagents and its dissociation behaviour. The neutral isoelectric point seemed reasonable since the ratio of acidic to basic amino acid residues is 1 (Schwenke et al., 1981).

The 12S globulin is actually a glycoprotein. It contains carbohydrate ranging in concentration from 0.5% to 13% and includes arabinose, galactose, glucose, inositol, glucosamine and mannose (Goding et al., 1970; Gill and Tung, 1978a; Mieth et al., 1983b). The complexing of sugars is partially caused by Maillard condensation reactions during oil extraction and meal desolventization. Thus, the amount of sugar associated with protein depends on the processing conditions and the extent of the Maillard reaction.

2. Association of Canola Proteins with Phytic Acid

The levels of phytate in canola have been reported to be 2.0 - 4.0% in the whole seed, 3.0 - 7.5% in the defatted meal and <1.0 - 9.8% in protein isolates (Alli and Houde, 1987; Thompson, 1990). The large variation for protein isolates mainly depends on the preparation method. The phytate in canola exists as metal salts. The major salts of phytic acid are Ca, Mg and K phytate. They are in the crystalline globoids (0.5 - 2.8μ in size) inside protein bodies in the cells of the radicle and the cotyledon.

The level of phytate solubilized from defatted meal under alkaline conditions (pH 7 or higher) is usually less than 10%. A relatively high quantity of phytate (> 50%) can be solubilized in the pH range of 4 - 6 (Alli and Houde, 1987; Zhou et al., 1990). This is because canola phytate is predominantly in the form of metal phytates, which are very soluble in this pH range. The interaction between phytic acid and protein has been reported to occur naturally in rapeseed but also takes place during processing including protein extraction (Thompson, 1990).

Studies of the interaction indicated that both the 1.7S albumins and 12S canola globulins could bind to phytic acid although the extent of binding and dependence on pH are not the same (Schwenke et al., 1986; Mothes et al.,1987; Schwenke et al., 1987).

The 1.7S albumin was soluble in the pH range of 2.0 - 10.0 but could be precipitated by adding phytic acid (Schwenke et al., 1986). At pH 3.0, where maximum protein precipitation occurred, only 0.8 mol phytic acid were bound to 1.0 mol of basic groups in the albumin molecule (Mothes et al., 1987). The 1:1 stoichiometric ratio (1.0 mol of phytic acid phosphate to 1.0 mol of basic groups in the protein) reported for the 12S globulin was not attainable at pH 3.0 for 1.7S albumin. (Schwenke et al., 1987). If excess phytic acid were added, the protein-phytic acid complex became soluble (Schwenke et al., 1986; Mothes et al., 1987). However, complete solubilization did not occur even in the presence of extremely high levels of phytic acid (Schwenke et al., 1987).

At pH values higher than 5, the formation of soluble phytic acid-protein complexes, resulted primarily in distinct protein dimers although there was evidence for

the formation of larger oligomers (Mothes et al., 1987). This doubling of the molecular mass in the presence of phytic acid in the pH range of 6.0 - 8.0 gave a fraction with a mean Stoke's radius of 2.3 - 2.9nm; the minor oligomer fraction had an average Stoke's radius of 15 - 35nm. The dimer was stable at low and medium ionic strength ($\mu \leq 0.2$) and contained 0.08g phytic acid bound per g albumin. The heat stability of the albumin solution was affected by the amount of phytic acid present. Phytic acid-free albumin solution underwent aggregation and coagulation on heating to 50°C or higher; but, in the presence of phytic acid, heat induced aggregation was inhibited. Upon heating, the radius of the dimer remained constant whereas the oligomers' radius increased slightly. In addition, the secondary structure of the albumin did not change after heating (Mothes et al., 1987). No binding of phytic acid with the albumin was observed in the pH range of 9.5 - 10.0.

The 12S globulin also formed an insoluble complex with phytic acid in the pH range below isoelectric point. Using the turbidimetric titration method, the amount of bound phytic acid was found to increase with decreasing pH until the 1:1 stoichiometric ratio was achieved at pH 3.0 (Schwenke et al., 1986). The precipitation yield at this pH was found to be 100% (Mothes et al., 1987; Schwenke et al., 1987). Unlike the albumin, there was no solubilization of this complex even where excess phytic acid was added (Schwenke et al., 1987). At pH values lower than 3.0, excess binding of phytic acid took place. The determined pH of "zero-binding" was close to the isoelectric range of pH 6.3 - 6.5. The results were slightly different when obtained by chemical analysis where maximum precipitation of the complex was only 95% at pH 3.0, and the binding ratio

was 1.18 mol phytic acid phosphorus to 1.0 mol basic groups in the globulin, higher than the expected 1:1 stoichiometric ratio (Schwenke et al., 1987).

3. Functional Properties of Canola Protein Products

a. General functional properties. The use of protein in foods largely depends on its functional properties such as solubility in water or salt solutions, water and fat binding, oil emulsification, viscosity and gelation. Bland flavours and light colour are also important sensory characteristics for most food grade protein products. In most of the studies of canola protein materials, soy protein products are often used as models for comparison.

Rapeseed flour was found comparable to soybean flour in term of water absorption (Sosulski et al., 1976). In comparison, the rapeseed flour had higher nitrogen solubility, fat absorption, oil emulsification, whippability and foam stability, but had poor gelling properties. The concentrates and isolates prepared by alkali extraction of rapeseed both had better water and fat holding capacity, while the isolates also had better oil emulsification and whipping characteristics than the corresponding soy protein products. Despite the superior functional properties, undesirable sensory characteristics such as the greenish brown colour persisted for the canola products.

Protein concentrates prepared using 2% sodium hexametaphosphate were found to have good nitrogen solubility, fat absorption, emulsification, and whipping capacity, but poor water absorption and gelling properties (Thompson et al., 1982). When the concentrate was used as a meat extender (at 3.4%) in wiener, the emulsion stability of the

wiener was increased. If used in meat patties, the addition of the concentrate reduced shrinkage, increased cooking yield and tenderized texture. Meringue made from 9% protein concentrate showed poor foam stability when compared with the meringue made from egg white. All of these food products, however, had poor sensory evaluation in terms of flavour and colour.

In a study using 5% replacement of wheat flour in bread baking, protein isolates obtained by water, HCl and NaOH extractions, decreased loaf volume by 10 to 20% (Kodagoda et al., 1973). With the addition of 0.5% emulsifier, the loaf volume was restored or even increased. In a whipping test, 3% replacement of egg white decreased foam volume for all products, except the HCl extracted isolate. All water, HCl and NaOH extracted protein isolates improved foam stability. Moreover, the water extracted protein isolate had the highest emulsification capacity, and the HCl extracted protein isolates had remarkable emulsifying stability.

Canola protein isolates extracted by countercurrent alkali extraction-isoelectric precipitation exhibited lower nitrogen solubility and moisture adsorption, even though oil adsorption was higher than the corresponding soy protein isolates (Dev and Mukherjee, 1986). Compared with the soy isolates, the canola isolates had similar or higher emulsifying capacity and emulsion stability.

Compared with soybean proteins products, canola products were characterized by overall favourable foaming capacity and foaming stability (Dev and Mukherjee, 1986). Moreover, canola products demonstrated similar or higher emulsifying activity and emulsion stability. Low-phytate canola products had better emulsifying properties then

their high-phytate counterparts, but the level of phytic acid had little effect on the foaming properties.

b. Thermal gelation properties. Rapeseed protein isolates prepared by alkali extraction-isoelectric precipitation generally have poor gelation characteristics. These gelling properties were first demonstrated in 1978 (Gill and Tung, 1978b). Using rapeseed protein extracted at pH 9.2, dialysed against running water at 4°C, concentrated by pervaporation, and dialysed against universal buffers at different pH values, thickening could be measured for a 1% protein dispersion, and gelation occurred at 4.5% protein level if the pH were equal to or higher than 4. The required protein level for gelation, 4.5%, was lower than the level needed for soy proteins. At this protein level, the rapeseed protein dispersions were readily self-associating on heating to form gels. Gel strength increased as pH and ionic strength increased. The ionic and intermolecular disulfide bonds were believed only to have a minor role in network formation. However, both covalent and noncovalent forces were involved in the gelation. Protein-carbohydrate interactions might also have an effect on the characteristics of the gel formation due to the present of a carbohydrate moiety in the 12S globulin. In a subsequent study, gels were formed only at high pH values (≥9.5) or after succinylation (Paulson and Tung, 1989). Forces involved in gel formation and stability were identified as hydrophobic interactions and hydrogen bonds. Using the isolate prepared by salt extraction and precipitation by dilution procedure, gelation was reported at a protein concentration of 6% (Léger and Arntfield, 1993). The gels prepared at alkaline pH values were stronger than the gels prepared under acidic conditions. Hydrophobic forces and electrostatic

interaction were found responsible for establishment of the gel network. Once the gel network formed, disulphide bonds, hydrogen bonds and electrostatic interactions contributed to gel stabilization and strengthening.

Generally, the rapeseed/canola proteins products are functional in foods and, in some instances, properties are even superior to those exhibited by soy protein products. Furthermore, the methods used for producing rapeseed/canola protein materials can adversely affect their functional properties. However, the investigations on the functional properties of rapeseed/canola proteins are not yet conclusive. More information on the influence of naturally present antinutritional factors is required.

III. MATERIALS AND METHODS

A. Materials

1. Bovine Serum Albumin

Bovine serum albumin (BSA), Fraction V fractionated by cold alcohol precipitation, was purchased from the Sigma Chemical Co. Ltd. (St. Louis, Missouri, USA). It was used to form a model system for studying thermally-induced gelation of proteins under the influence of phytic acid.

2. Canola 12S Globulin

A defatted, and also degummed canola meal was provided by the Canamera Food (Russell, Manitoba) and subjected to a modified protein micellar mass technique (Burgess, 1991). This procedure involves salt solubilization followed by precipitation through a reduction in ionic strength. The scheme of the procedure was shown in Fig. 6. The 12S globulin isolated under these mild conditions retains most of its native conformation. The isolate contained the 12S fraction and was electrophoretically homogeneous (Léger, 1992). The composition of the protein isolate was analyzed and reported to contain 89.08% protein, 3.94% fat, 1.31% phenolics, 0.310% glucosinolates and 0.376% phytic acid.

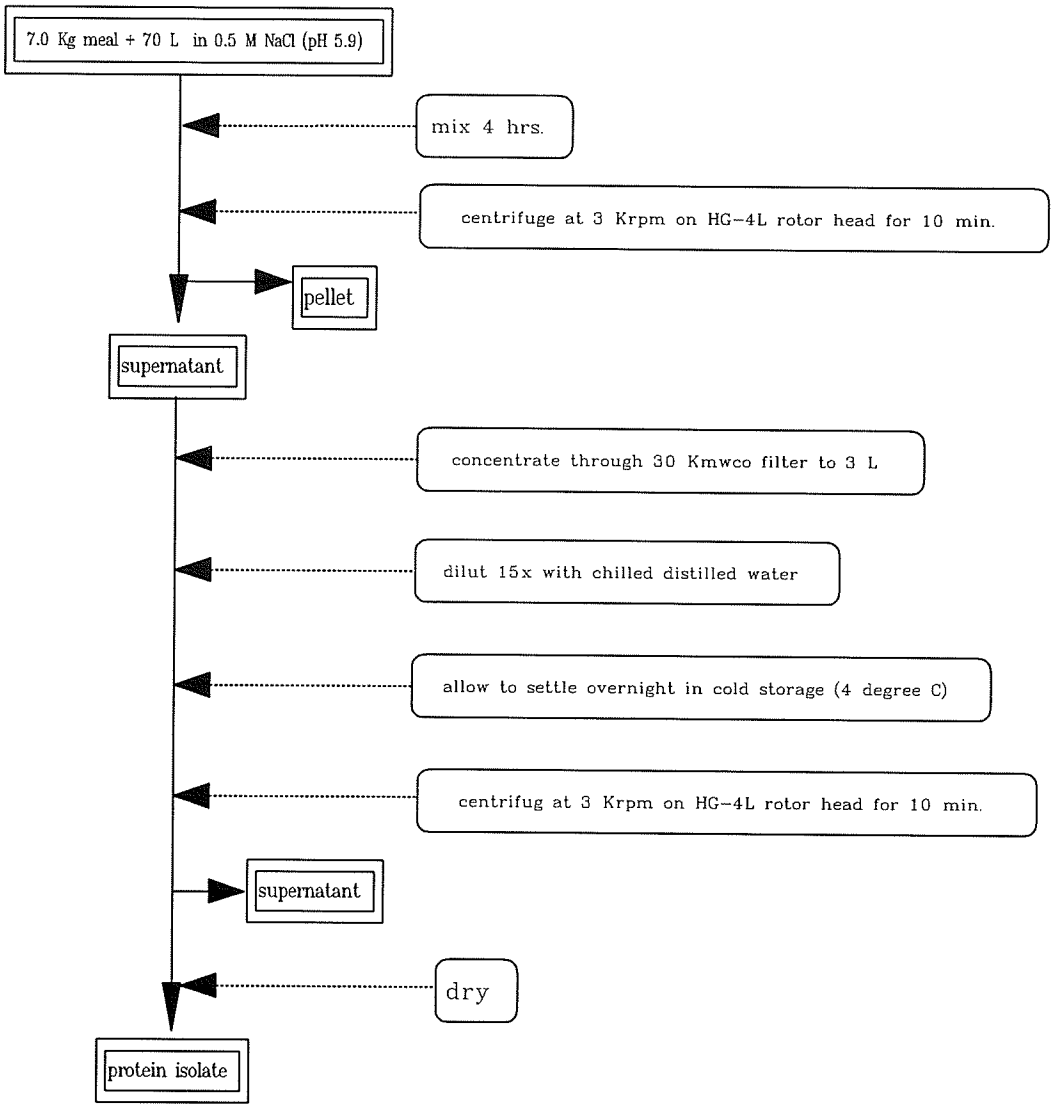


Figure 6. The Procedure of the Isolation of Canola Protein Isolate (Burgess, 1991)

3. Other Reagents

Phytic acid, in the form of a sodium salt, was obtained from the Sigma Chemical Co. Ltd. (St. Louis, Missouri, USA). It was 97% pure, contained 15% moisture by weight and had 12 sodium/mole. In Table 3, other chemicals used in the experiment and their sources are listed. All chemicals used were reagent grade.

Table 3: Chemicals Used

Chemicals	Manufacturers
AG®1-X8 Anion Exchange Resin 200-400 mesh chloride form	Bio-Rad Laboratories (Mississauga, Ontario)
Hydrochloric acid	Anachemia Science Co. Ltd. (Winnipeg, Manitoba)
Barbital	BDH Inc. (through VWR CaLab Co. Ltd., Edmonton, Alberta)
Boric acid	Sigma Chemical Co. Ltd. (St. Louis, Missouri)
Calcium chloride	Fisher Scientific Co. Ltd. (Edmonton, Alberta)
Hydrated ferric chloride	Fisher Scientific Co. Ltd. (Edmonton, Alberta)
Sodium chloride	Mallinckrodt Co. Ltd. (through Anachemia Science Co. Ltd.)
Sulphosalicylic acid	Mallinckrodt Co. Ltd. (through Anachemia Science Co. Ltd.)
Sodium hydroxide	Mallinckrodt Co. Ltd. (through Anachemia Science Co. Ltd.)
mono-Potassium phosphate	Mallinckrodt Co. Ltd. (through Anachemia Science Co. Ltd.)
Citric acid	Mallinckrodt Co. Ltd. (through Anachemia Science Co. Ltd.)

B. Methods

1. Sample Preparation

The different environments used to examine the extent of protein phytic acid binding and thermal gelation involving BSA and the canola 12S globulin are list in Tables 4 and 5 respectively.

Table 4: Environments Used to Study Protein Binding and Thermal Gelation of BSA

Variable	General Conditions	Values Examined
pH	0.15M NaCl in buffer	4, 5, 6, 7, 8, 9
Phytic acid	0.15M NaCl in buffer	1, 2, 3, 5 %*
Ca^{2+}	0.15M NaCl in buffer	0, 0.005, 0.01 M

* based on the weight of protein

Table 5: Environments Used to Study Protein Binding and Thermal Gelation of the Canola 12S Globulin

Variable	General Conditions	Values Examined
pH	in buffer	5, 7, 9
Phytic acid	in buffer	0, 2, 5 %*
Ca^{2+}	in buffer	0, 0.01 M

* based on the weight of protein, no zero value for binding studies

a. Preparation of Robinson universal buffer. Because the interaction between phytic acid, cations and proteins is pH dependent, buffers were used to control the pH in these experiments. Robinson Universal Buffer was chosen in this experiment since it could provide a very wide range of pH, 2.38 - 12.10 (Britton and Robinson, 1931; Perrin and Dempsey, 1974). First of all, a 0.15M NaCl solution was prepared with distilled water (17.5328g of NaCl in 2000mL). The stock solution I of the buffer was made by combining 3.893g dihydro monopotasium phosphate, KH_2PO_4, 6.008g citric acid, 1.769g boric acid and 5.346g diethyl barbituric acid in 1L NaCl solution. Stock solution II was prepared by dissolving 7.999g NaOH in 1L NaCl solution. To 100mL of stock solution I, xmL of stock solution II was added to adjust the pH level of the buffer. In Table 6, the required amounts of stock solution II at different pH levels examined are listed. In the studies of canola 12S globulin, the buffers contained no NaCl, as the presence of salt interfered with gel formation.

Table 6: Required Amount of Stock Solution II at Different pH Levels

pH	mL of Stock Solution II
4	15.5
5	27.1
6	38.9
7	50.6
8	63.7
9	72.7

b. Preparation of sample for gel rheological studies. Samples of BSA and the canola 12S globulin for rheological analysis consisted of 9.1% (w/w) protein prepared in Robinson Universal buffer at various phytic acid and Ca^{2+} ion levels. Minor pH adjustments were made using drop-wise addition of 6M HCl and 4M NaOH monitored with a Radiometer 26 pH meter (Bach-Simpson Ltd., London, ON). The solutions were then allowed to sit for 30 minutes, and the pH rechecked. If necessary, the pH was readjusted.

c. Preparation of sample for protein binding studies. The samples for the binding study consisted of 1.0mL of a protein dispersion plus 1.0mL of a ligand solution for each sample. The protein dispersions contained 9.1% (w/w) protein in different

concentrations of Ca^{2+} dissolved in Robinson Universal Buffer of the appropriate pH. The ligand solutions were also prepared by dissolving the appropriate quantity of phytic acid in Robinson Universal buffer at different pH levels. The pHs of solutions were adjusted using drop-wise addition of 6M HCl and 4M NaOH to correct for any minor shifts in pH due to the dissolved material. The solutions were allowed to sit for 30 minutes, the pH was rechecked and adjusted if necessary prior to use.

2. Small Amplitude Oscillatory Rheology

Approximately 1.0mL of protein sample was pipetted onto the lower plate of a 30mm parallel plate configuration. The upper plate was then lowered onto the sample so that a 1mm gap was obtained. In order to prevent drying of the sample during the heating sequence, a masking tape well was formed around the outer edge of the cylinder holding the lower plate. Paraffin oil, Saybolt viscosity at 100°F 125/135 (Fisher Scientific), was added to fill the well until it just covered the upper plate. The protein solutions were heated to 95°C at 2±0.2°C/min and then cooled to 25°C at the same rate using the Bohlin rheometer (Bohlin Rheologi, Inc., Lund, Sweden), operated in oscillation mode. The dynamic test characteristics including G', the storage modulus, and G", the loss modulus were assessed every minute. Input strain amplitude for dynamic analysis was 0.02 and frequency used for the thermal scans was 0.1Hz. The sensitivity of the measurement was determined by a calibrated 93.2gcm torque bar. Upon the completion of the thermal scan, final network characteristics were evaluated as a function of oscillatory frequency over the range (0.05 to 10Hz) at a constant temperature of 25°C.

In addition to the G' and G" moduli which are monitored automatically, the tangent delta (tan δ =G"/G'), a measure of the energy lost due to viscous flow compared to the energy stored due to elastic deformation in a single deformation cycle, was calculated from the data obtained in the dynamic test. As plots of log G' and log G" as a function of oscillatory frequency were parallel, values at a frequency of 1Hz from frequency sweep were used for further comparison.

3. Equilibrium Dialysis for Protein Binding Study

Binding behaviour of phytic acid with proteins in different calcium ion concentrations was determined using the Spectra/Por® 5-Cell Equilibrium Dialyser (Spectrum®, Texas). The standardized dialysis cells are made of Teflon. They are machined so that the cell halves seal the dialysis membrane dividing the inner chamber of the cell into two equal compartments (each with working volume of 1.0mL), one on each side of the membrane. Five Teflon cells, separated by stainless spacers, were stacked in a cell carrier. The assembled cells in the carrier were then mounted onto the drive unit and rotated at 20 rpm about an axis perpendicular to the membrane in a water bath at 25°C. As a result, five experiments could be performed simultaneously.

In this equilibrium dialysis study, Spectra/Por® 4 membrane discs with MWCO of 12,000-14,000 were used. The precut membrane discs were first soaked in distilled water for at least 15 minutes. Then the discs were rinsed and drained. After that, they were transferred and soaked in 30% ethyl alcohol for another 20 minutes. The discs were again rinsed in distilled water to remove the alcohol. To condition the membrane discs

for the experiments, they were placed in the buffer for 15 minutes just prior to use.

The protein solution (1mL) was introduced into one cell compartment and the ligand solution (1mL) into the other compartment by using a syringe. Finally, all the filling ports were closed with stoppers. The protein solution in different Ca^{2+} concentrations at various pH levels was allowed to react with phytic acid for a time period to ensure equilibrium had been reached. To determine the required time of equilibrium, controls without protein were run in different experimental conditions.

Following dialysis, the concentration of phytic acid remaining in the ligand side of the dialysis system was determined by a simple method (Latta and Eskin, 1980). This method involved a clean up with an anion-exchange resin followed by a reaction with ferric chloride/sulfosalicylic acid, the color reaction of which were measured spectrophotometrically. All samples in the binding studies were assayed by this method for phytic acid content. The concentration of the bound phytic acid was calculated by subtracting the amount of remaining free phytic acid in the samples from the amount of the corresponding controls.

4. Statistical Analysis

A full factorial design was used to analyze the experiments in this study after consultation with the statistical advisory service (University of Manitoba, Winnipeg, Manitoba). All statistical analysis was performed using Statistical Analysis System (SAS) under Unix. Statistical differences were determined using an Analysis of Variance in conjunction with a Duncan's Multiple Range Test. The results obtained at each individual

environmental combination are provided in appendices as means and standard deviations. Duplicate samples were carried out to confirm all the measurements in the experiments.

IV. RESULTS AND DISCUSSION

A. Protein Binding Studies

The effects of pH, phytic acid and calcium on the binding of phytic acid to bovine serum albumin (BSA) and the canola 12S globulin were investigated and assessed in this binding study. Based on the results of statistical analyses, main effects and interactions of all these factors were observed. A summary of all statistical analyses is given in the Appendices. In Appendix A and A1 to A10, the results of analyses for BSA are included; in Appendix D and D1 to D3, the results of the 12S globulin are shown.

In the following sections, the main effects of pH and the concentrations of phytic acid and the calcium ion on the phytic acid binding to the proteins will be examined first, followed by the effects of two-way and, finally, three-way interactions. Although the main effects, sometimes, will be masked by the effects of interactions, main effects should be examined first, followed by the interactions. This is because each interaction essentially represents a modification of the main effect or a lower level interaction effect. Thus, without the consideration of the corresponding main effects, there will be no sensible interpretation.

1. Main Effect of Individual Experimental Factor

a. Effect of pH Based on the evaluation of data (Table 7), the phytic acid binding to BSA and the canola 12S globulin was significantly higher at low pH values.

The highest levels of binding for BSA and the canola 12S globulin were observed at pH 4 and 5 respectively. The isoelectric point of BSA is 4.7 (Prattley et al., 1982). The binding to BSA decreased as the pH level increased up to pH 7 where the lowest mean value was observed; values at pH 8 and 9 were slightly higher. Binding to the 12S globulin, on the other hand, decreased significantly as pH increased. It appeared that the binding of phytic acid to the proteins was intensified at pH values lower than their isoelectric points. In general, the canola 12S globulin had a higher mean binding extent than BSA. The fact that there was a low level of phytic acid (0.376%) associated with the canola isolate did not seem to impair its potential for binding phytic acid.

Table 7: Effect of pH on Phytic Acid Binding (mole of phytic acid/mole of protein) BSA and Canola 12S Globulin

pH	Binding Extent of BSA (mol./mol)	Binding Extent of Canola 12S Globulin (mol./mol)
4	6.591a	---
5	2.301b	15.18a
6	0.02790cd	---
7	-0.3727e	4.091b
8	0.1679c	---
9	-0.08260d	-5.234c

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

b. Effect of phytic acid concentration In Table 8, the effect of phytic acid

concentration on the binding to BSA and the 12S globulin is shown. Binding to the canola 12S globulin increased significantly as the percentage of phytic acid increased from 2 to 5%. For BSA, there was no difference in phytic acid binding from 2% to 5% phytic acid but the mean values in this percentage range were significantly greater than the mean value for 1% phytic acid. In addition, the range of the mean values for all BSA samples was comparatively smaller than the one for the canola 12S globulin.

Table 8: Effect of Phytic Acid Concentration on the Binding of Phytic Acid to BSA and Canola 12S Globulin

	Binding Extent (mole of phytic acid/mole of protein)	
% Phytic Acid	BSA	12S Globulin
1	1.194b	---
2	1.501a	2.369b
3	1.557a	---
5	1.503a	6.989a

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

c. Effect of calcium For both BSA and the canola 12S globulin, the presence of calcium ions did not promote the binding of phytic acid to the proteins, as indicated in Table 9. Overall, as the concentration of the calcium ion increased, the mean binding values significantly decreased for both proteins. The highest values for binding were obtained in the absence of the calcium ion. As the role of calcium in the binding of

phytic acid to protein has been shown to be pH dependent, this observation may reflect the higher level of binding at low pH values where calcium should not be a factor (Saio et al., 1968; de Rham and Jost, 1979; Cheryan, 1980; Prattley et al., 1982; Grynspan and Cheryan, 1989). The interaction between pH and calcium concentration should clarify this result. The range of mean values for the canola 12S globulin was again greater than the range for BSA.

Table 9: Effect of Calcium on the Binding of Phytic Acid to BSA and Canola 12S Globulin

	Binding Extent (mole of phytic acid/ mole of Protein)	
Molar of Ca^{2+} ion	BSA	12S Globulin
0	2.209a	7.147a
0.005	1.142b	---
0.01	0.965c	2.210b

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

2. Effects of Interactions of Experimental Factors

a. Interaction between pH and phytic acid concentration There were significant interactions between pH and phytic acid concentration in term of the binding to both BSA and the canola 12S globulin (Appendix A and D). In Fig. 7, the interaction effect on the binding to BSA is shown. At pH 4, the highest values of binding were

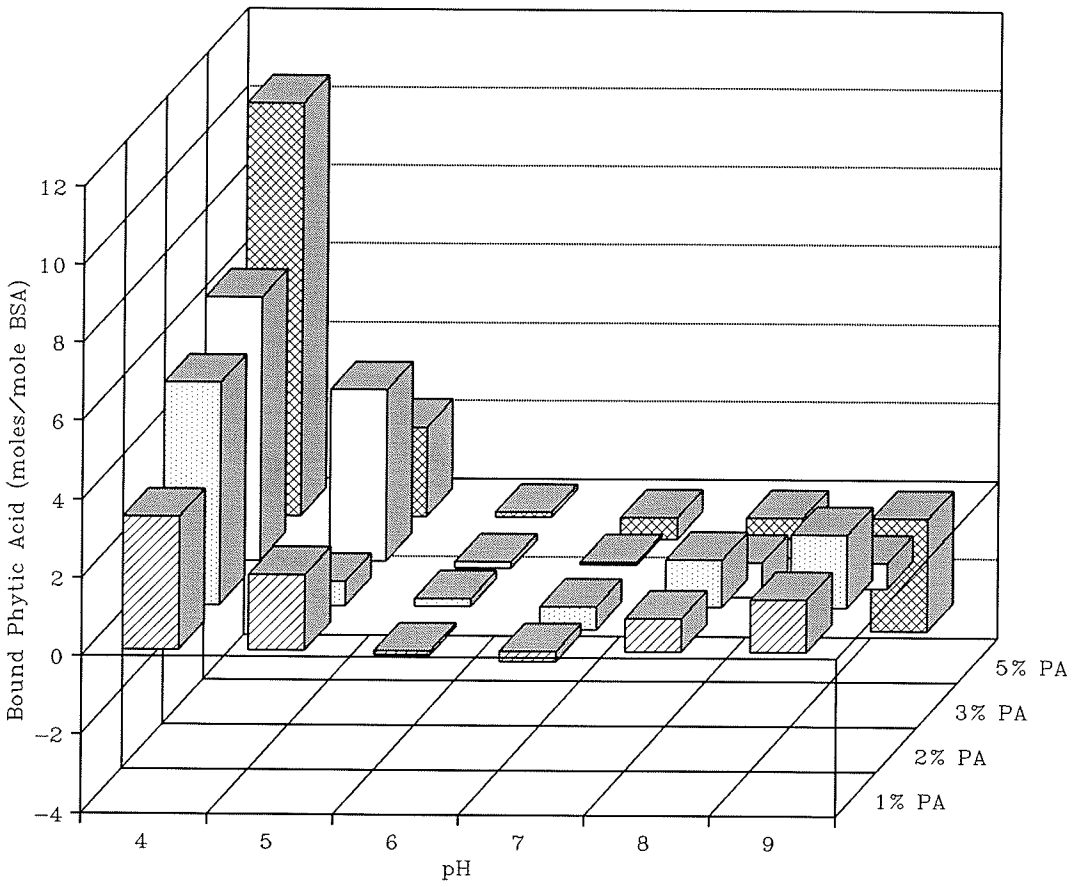


Figure 7: Effect of the Interaction between pH and Phytic Acid (PA) on Phytic Acid Binding to BSA

observed for all levels of phytic acid. In addition, more binding occurred at pH 4 if the sample contained more phytic acid. The highest mean value was obtained in 5% phytic acid at pH 4. However, the mean values of the binding extent at pH 5 did not always have the second highest values although this was the trend observed with the main effect of pH. For instance, the second highest value in 2% phytic acid was not at pH 5 but at pH 9. Moreover, the mean values at pH 5 and higher did not increase as the percentage of phytic acid increased. It is the behaviour at these pH values that is responsible for the similar binding behaviour between 2 and 5% phytic acid when looking at main effects. In a comparison of all pH levels, pH 7 had the lowest mean values in all levels of phytic acid. In fact, the mean values were all negative at this pH. The negative values might be due to the experimental error. In the presence of calcium, it is possible that phytic acid in the controls was tied up by the calcium ion and gave lower values. After subtracting the phytic acid values in the samples containing protein from these controls to determine the amount of bound phytic acid, negative values were obtained, and have been reported as such.

The effect of the interaction between pH and phytic acid concentration on the binding of phytic acid to canola 12S globulin is shown in Fig. 8. The mean values decreased as pH value was increased regardless of phytic acid concentration. This corresponds to the trend seen for the main effect. The binding in 5% phytic acid was higher than in 2% at pH 5 and 7. At pH 9, however, binding of phytic acid to the 12S globulin samples gave similar results with 2 and 5% phytic acid.

b. Interaction between phytic acid and calcium concentrations The interaction

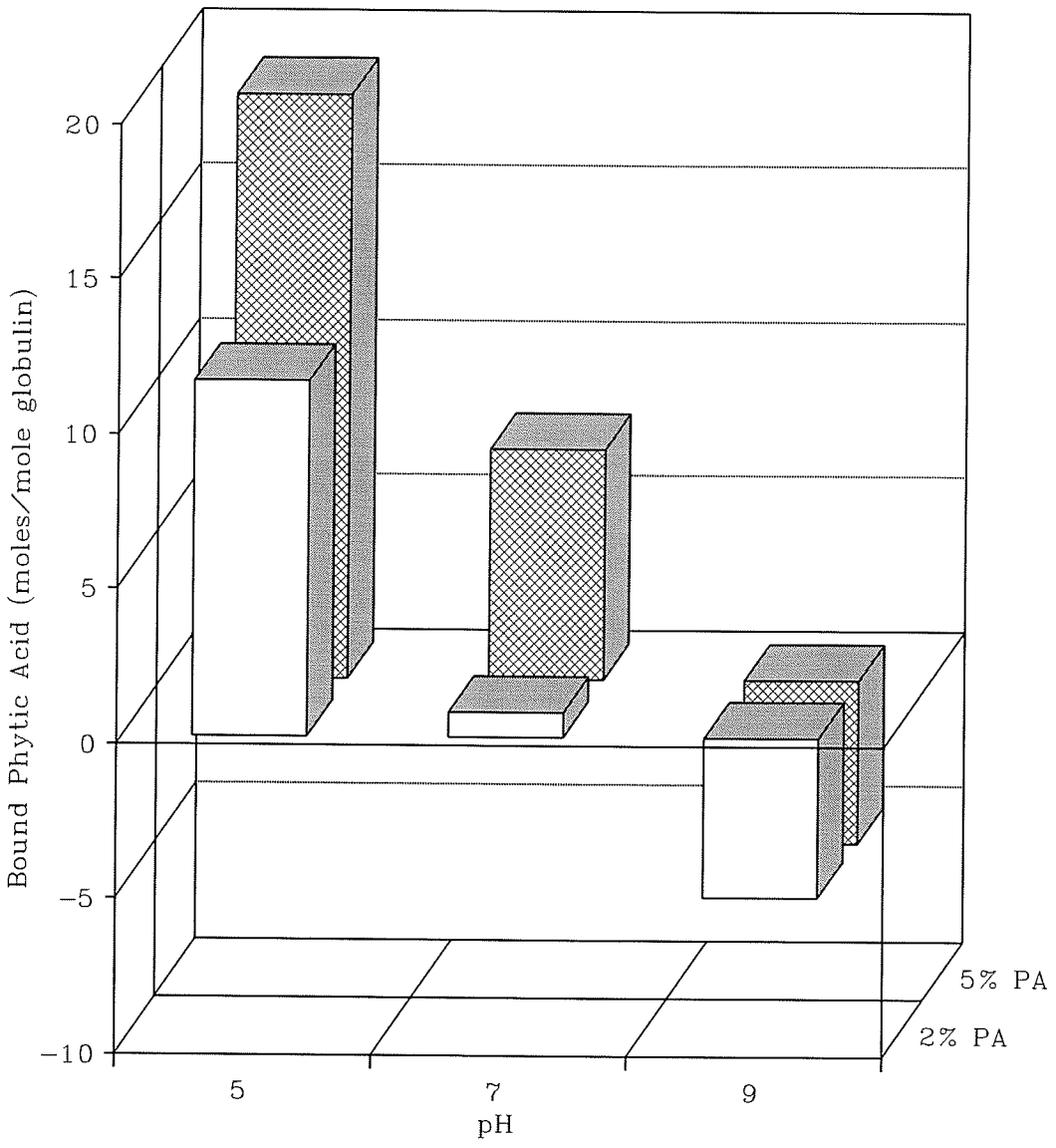


Figure 8: Effect of the Interaction between pH and Phytic Acid (PA) on Phytic Acid Binding to Canola 12S Globulin

between phytic acid and calcium concentrations was determined to have significant effects on the binding of phytic acid for both BSA and the canola 12S protein (Appendix A and D). In Fig. 9, the effect of the interaction between phytic acid and calcium concentrations on the binding to BSA is shown. At most levels of phytic acid, the presence of the calcium ion discouraged the binding of phytic acid to BSA. The exception was in 3% phytic acid, where increasing calcium ion concentration induced more binding of phytic acid to BSA. In the presence of the calcium ion, the greatest binding was observed in 3% phytic acid while lowest values occurred in 5% phytic acid. On the other hand, when the BSA samples contained no calcium ion, binding was greatest in 5% phytic acid and the lowest in 3% phytic acid. These observations are different from the main effects of the concentrations of phytic acid and calcium which where an increase in the concentration of phytic acid increased binding but an increase in the concentration of calcium decreased the binding. Clearly, the presence of calcium limited the contribution that phytic acid concentration could make to the binding of phytic acid to BSA.

For the 12S globulin, the effect of the interaction is shown in Fig. 10. As the concentration of phytic acid increased, the binding increased in the presence and absence of the calcium ion. The presence of calcium, however, discouraged phytic acid binding to the globulin. These trends are similar to those observed for the main effects although there is a significant interaction. The interaction reflects the magnitude of the differences between the binding at different levels of calcium and phytic acid.

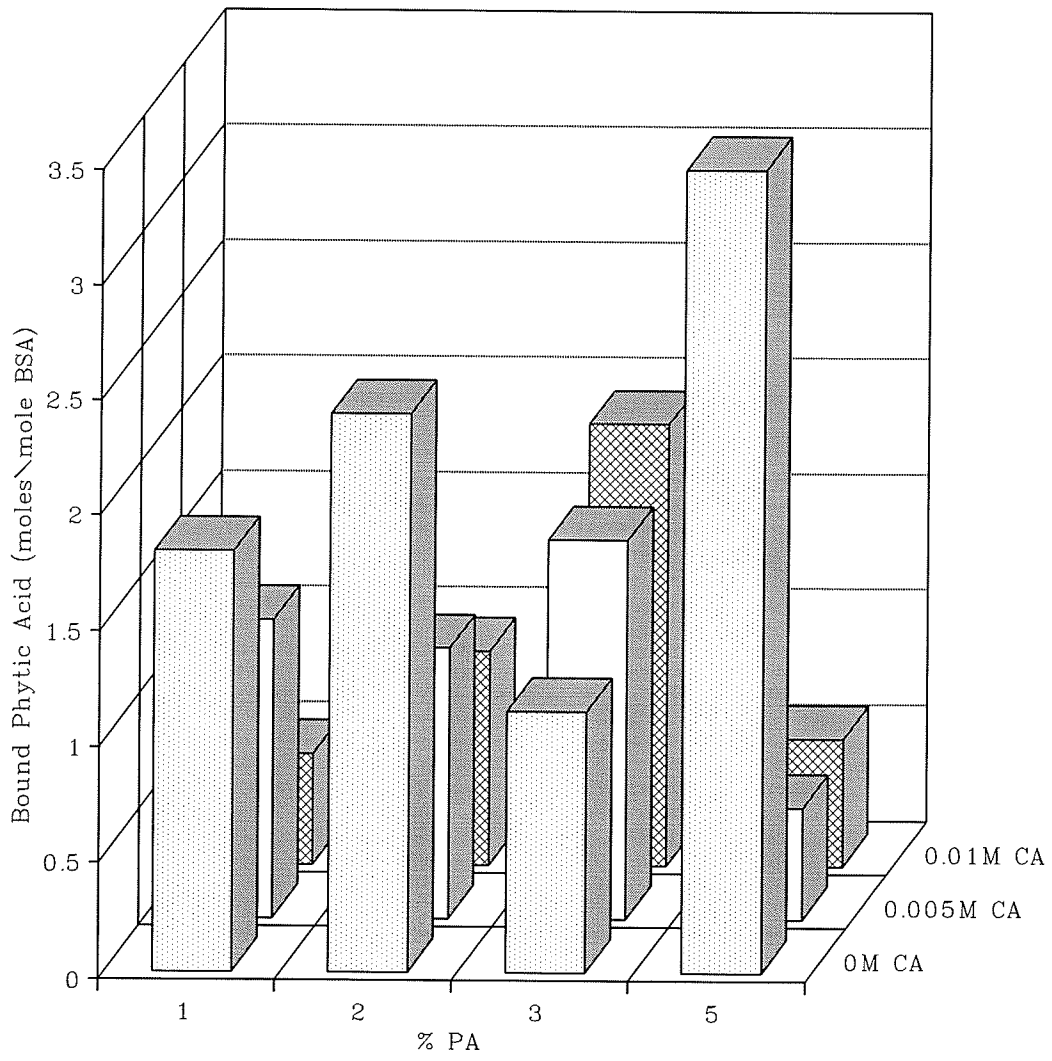


Figure 9: Effect of the Interaction between Phytic Acid (PA) and Calcium (CA) on Phytic Acid Binding to BSA

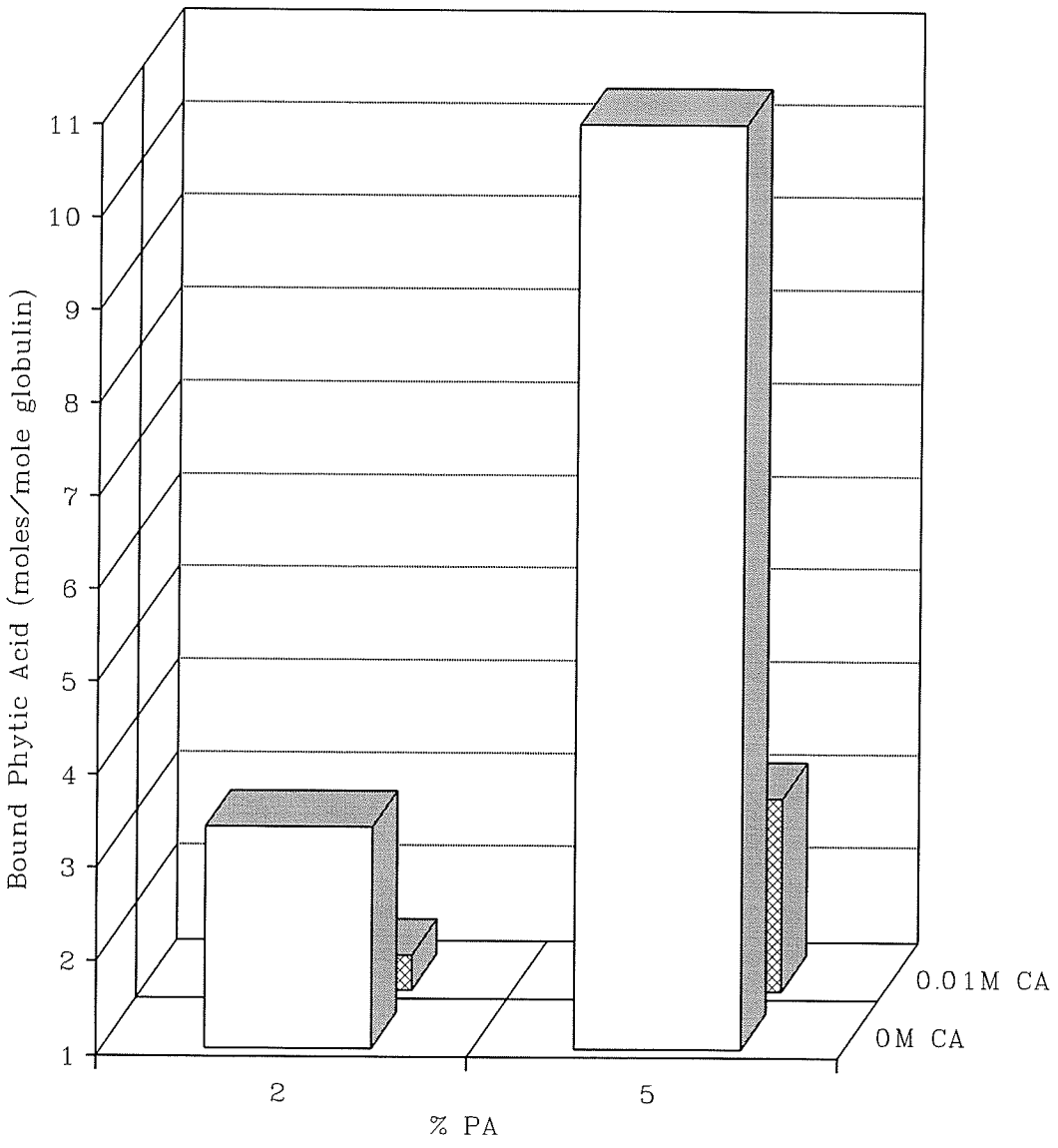


Figure 10: Effect of the Interaction between Phytic Acid (PA) and Calcium (CA) on Phytic Acid Binding to Canola 12S Globulin

c. Interaction between pH and calcium The binding of BSA under the influence of the interaction between pH and calcium is shown in Fig. 11 (Appendix A). The highest mean values were observed at pH 4 for all levels of the calcium ion. At this pH value, the presence of the calcium ion increased the extent of binding. At pH 5, increasing the calcium ion concentration resulted in less phytic acid binding. At higher pH values, however, only the samples prepared in the absence of the calcium ion had positive binding values indicating there was no phytic acid binding in the presence of the calcium ion. The main effect of calcium is, therefore, modified by a pH-calcium interaction. The presence of calcium does not always discourage the binding, just at pH values above the isoelectric point.

The interaction between pH and calcium had no significant effect on the binding of phytic acid to canola 12S globulin (Appendix D).

d. Interaction among all experimental factors Statistically, the simple effects of factors in all experimental treatments, with the consideration of the corresponding main effects and the lower level interactions, shall provide the basis for interpretation if the three-way interactions are significant. In other words, the experimental results shall be interpreted in terms of mean binding extent for all combinations from all three factors.

There are significant 3-way interactions, among pH and the concentrations of phytic acid and calcium, on phytic acid binding to both BSA and the canola 12S globulin (Appendix A and D). The simple effects of pH, phytic acid and calcium concentrations on the binding are demonstrated in Appendix A1 to A10 for all experimental treatments. The effect of this interaction on the binding to BSA is shown in Fig. 12. The binding

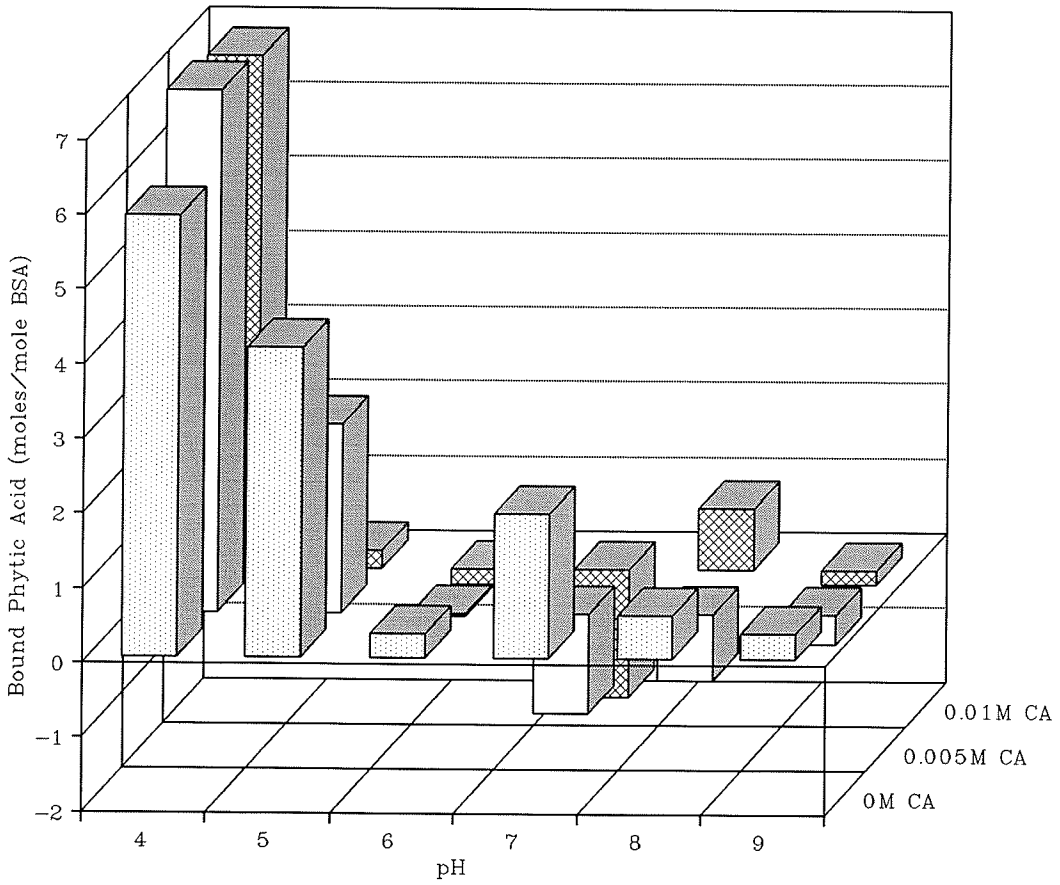


Figure 11: Effect of the Interaction between pH and Calcium (CA) on Phytic Acid Binding to BSA

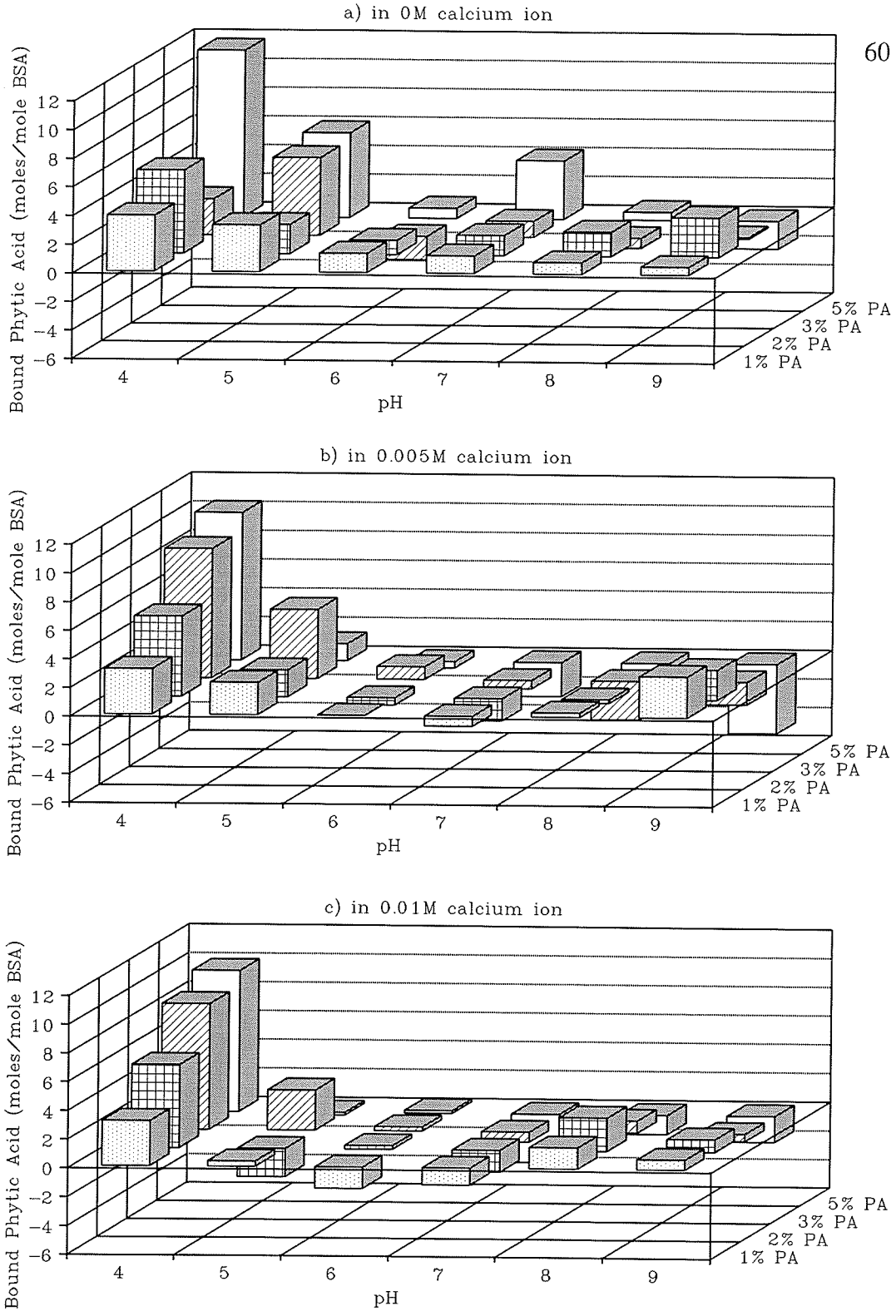


Figure 12: Effect of pH on Phytic Acid (PA) Binding to BSA
a) in 0M, b) in 0.005M and c) in 0.01M calcium ion

activities under various experimental treatments were very different from each other. The mean values at pH 4 were always greater than the values at other pH values in all concentrations of phytic acid and calcium ion. The fact that this pH is below the isoelectric point of BSA would create a situation where direct electrostatic interaction between the protein and phytic acid would be encouraged (Cheryan, 1980; Prattley et al., 1982; Grynspan and Cheryan, 1989). This could account for the greater binding. At this pH, binding increased with increased concentrations of phytic acid (except in 3% phytic acid with no calcium). This would again reflect a direct binding between phytic acid and BSA, where the available sites on BSA had not been satisfied even at the 5% phytic acid level. This agrees with the 2-way interaction between pH and phytic acid. With the exception of the sample in 3% phytic acid, the presence of calcium had no impact on phytic acid binding. This was expected as the direct binding does not require calcium and agrees with data from the 2-way interactions between calcium and phytic acid. At pH 5, the impact of the 3-way interaction is more apparent. The 2-way interaction of pH and phytic acid indicated maximum in binding at 3% phytic acid. The 3-way interaction indicates that this trend holds true only in the presence of calcium. With no calcium, increasing phytic acid increased the level of binding. However, the calcium ion was found to discourage the extent of binding regardless of the concentration of phytic acid. Reduction in bound phytic acid was observed previously in the presence of calcium at low pH level (Okubo et al., 1976; Prattley et al., 1982). The observation can be attributed to competition between protein and calcium for binding sites on the phytic acid molecule. BSA at pH 5 contained slightly more positive residues than the negative one. The

repulsion between BSA and phytic acid should not be strong enough to prevent contact and binding. Moreover, the α-NH_2 terminal groups and ε NH_2 of lysine, the preferable binding sites, are still protonated (Cheryan,1980). Thus, the binding mechanism would be a direct electrostatic binding.

At higher pH values, the binding was generally low and somewhat erratic regardless of phytic acid concentration or calcium level. These results are comparable to those observed with the 2-way interactions. This is unexpected based on the literature which indicated a ternary complex involving calcium, phytic acid and protein (Saio et al., 1968; Cheryan, 1980; Prattley et al., 1982; Grynspan and Cheryan, 1989). There is a lack of evidence of such a complex in this study. In order to have the formation of ternary complex, there should be unprotonated imidazole groups of histidine, the most probable binding sites, on the protein molecule. Since their pK value in general is 6 to 8 (Cheryan, 1980; Grynspan and Cheryan, 1989), it may explain why binding cannot be observed from the isoelectric point of protein to pH 6 or even 8. The increased binding with increased calcium ion seen with 3% phytic acid in the 2-way interaction of phytic acid and calcium resulted primarily from the data at pH 4 and 5 where there is a significant binding even though the values for the sample including 3% phytic acid and 0M calcium ion was uncharacteristically low.

In Fig. 13, the effect of the 3-way interaction on the binding of phytic acid to the canola 12S globulin is shown (Appendix D1 to D3). The mean binding to this globulin decreased as pH increased in all treatments as was seen for the main effects. The formation of phytic acid-globulin complex had been observed in the pH range (2 - 6)

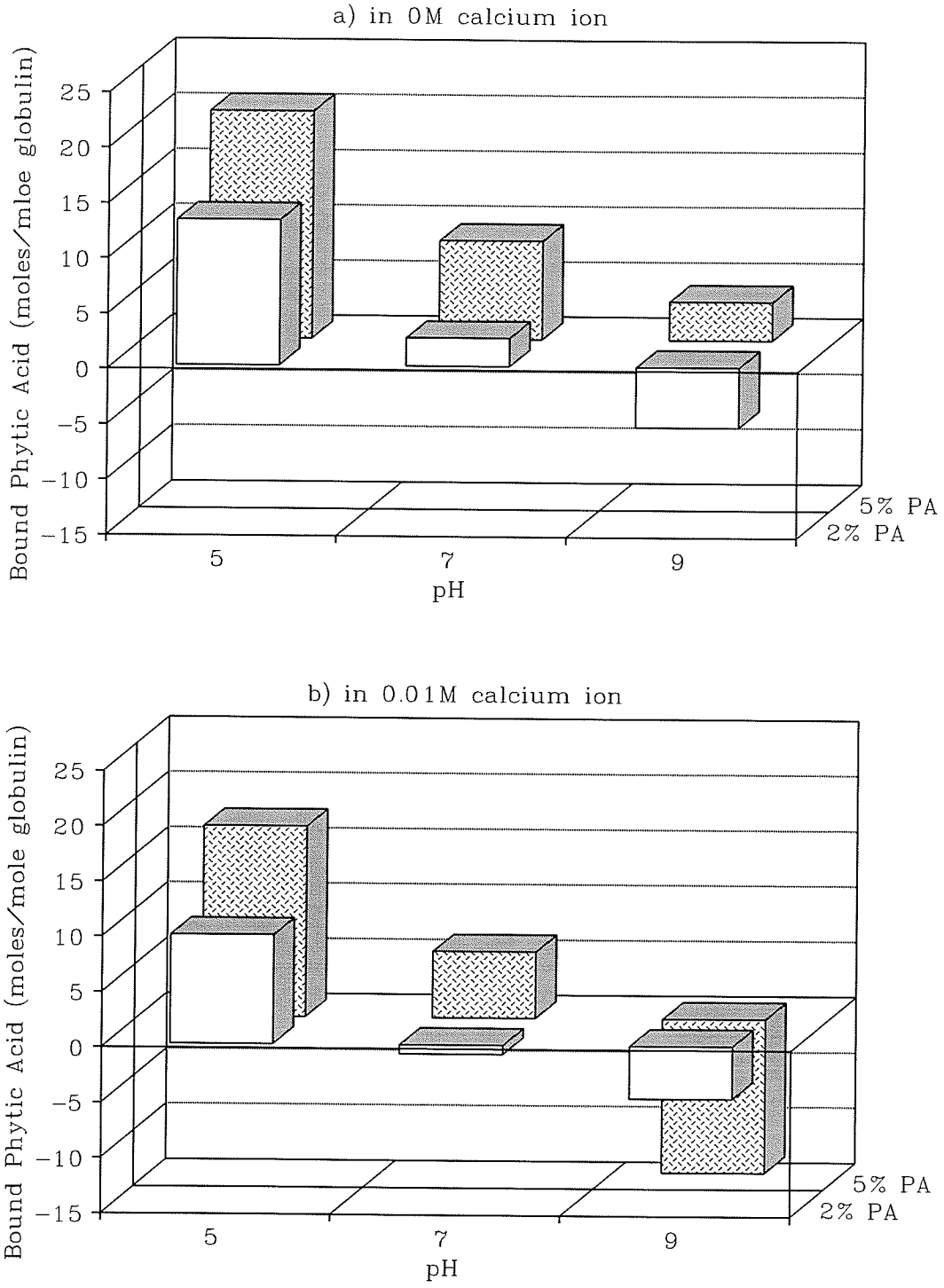


Figure 13: Effect of pH on Phytic Acid (PA) Binding to Canola 12S Globulin
a) in 0M calcium ion
b) in 0.01M calcium ion

below the isoelectric point (Schwenke et al., 1986; Schwenke et al., 1987; Mothes et al., 1987). The amount of phytic acid bound to the protein was found to increase with decreasing pH. The main effects of calcium and phytic acid concentrations and 2-way interactions of pH and phytic acid that have been reported previously were apparent at pH 5 and 7. It is the behaviour at pH 9 that was responsible for the 3-way interaction. At this pH, the effect of phytic acid concentration on the binding to the canola globulin was dependent on the calcium ion concentration. With no calcium, 5% phytic acid had more binding than 2% while with calcium, the reverse was true. The adverse effect of calcium should again be due to the competition between protein and the calcium ion for phytic acid especially at pH values below the isoelectric point.

For BSA and the canola globulin, pH seemed to have more impact on the phytic acid binding. The highest binding to BSA was achieved at pH 4. In case of the canola globulin, the highest binding can be achieved in 5% of phytic acid level at pH 5 or lower, without the presence of calcium. The reduction of the binding to protein with rising pH in the absence of calcium has been observed perviously (Prattley et al., 1982). When protein possesses more positively charged binding sites at the lower pH value, the negatively charged phytic acid binds to the protein readily without the help of multivalent cations. The decrease in binding at higher pH values is attributed to the repulsion of the phytic acid molecules by the increasingly negatively charged protein (Prattley et al., 1982).

BSA was shown to bind less phytic acid than canola 12S globulin. These differences could be explained by their molecular sizes, 66000 and 350000 g/mol

respectively. The molecule of the 12S globulin is about five times larger than the molecule of BSA. Thereby, there should have more binding sites on the globulin molecule. In addition, the bound phytic acid could crowd the surface of the molecule of BSA, and thus prevent further binding of phytic acid even though binding sites were not completely saturated.

For both BSA and the 12S canola globulin, there is a lack of evidence that the calcium ion can enhance the binding of phytic acid through the formation of a ternary complex. This is in contrast to the literature. Although many researchers claim the existence of a ternary complex, the observed increase in bound phytic acid can be caused by the formation of insoluble calcium phytate at alkaline pH (Prattley et al., 1982). In most of the solubility and binding studies using filtration technology, no attempt has been made to distinguish calcium phytate and the ternary complex. Additionally, some of these studies were done in extremely high calcium ion concentrations (Saio et al., 1968; Prattley et al., 1982; Grynspan and Cheryan, 1989). Thus, the lack of ternary complex formation in this study may also due to the low concentration of the calcium ion used.

B. Gel Rheological Studies

1. Effect of Individual Experimental Factor

a. Effect of pH on the storage modulus (G') By affecting ionization and the net electrical charge on the protein molecule, pH can influence the type of association that takes place following heat induced denaturation by altering the ratio of attractive and

repulsion forces among proteins (Paulson and Tung, 1988; Arntfield et al., 1990). In Table 10, the effects of pH on the storage modulus (G'), or network structure, of BSA and the canola 12S globulin gels are shown (Appendices B and E). The storage modulus was used to measure the rigidity of the gel: strong and rigid gels have higher values of storage modulus, and gels with poor network formation have lower values. The G' values for BSA gels were significantly lower at the two pH extremes, pH 4 and 9. The maximum value was obtained at pH 5 just above the isoelectric point, but significantly decreased as the pH increased to 9. At pH 5, close to the isoelectric point of BSA, attractive forces are expected to be high, causing a lumpy texture rather than a true gel network structure (Gill and Tung, 1978b). These are elastic and rigid localized aggregates. Thus, the observed storage moduli need not necessarily reflect the true gel strength. The 12S globulin gel had its lowest mean at pH 7, which is close to the isoelectric point. In contrast to the BSA gel, the G' values of the canola gel were significantly higher at the two pH extremes, pH 5 and 9, with the highest mean value occurring at pH 9. pH has previously been found to have significant impact on the rheology of the canola globulin gels (Léger and Arntfield, 1993), with rigid and elastic gels being formed at pH 9 or higher.

Table 10: Effect of pH on the Storage Modulus (G') of BSA and Canola 12S Globulin Gels

	G'	
pH	BSA Gel	12S Globulin Gel
4	1246e	---
5	38450a	47.51b
6	17330b	---
7	7813c	15.29c
8	5533d	---
9	2095e	120.8a

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

b. Effect of pH on the tan delta The effect of pH on the tan delta of the protein gels is shown in Table 11 (Appendices C and F). Lower tan delta values reflect increased elasticity in the gels. As the pH value increased, the tan delta for BSA gel significantly decreased but there was no significant difference from pH 7 to 9. The canola gels had a similar trend with the higher pH values giving smaller tan delta values. In both cases, high pH values, above the isoelectric point, seem to insure the elasticity of both protein gels. Similar results for the canola protein gels have been reported previously (Léger and Arntfield, 1993).

Table 11: Effect of pH on the tan Delta of BSA and Canola 12S Globulin Gels

	tan Delta	
pH	BSA Gel	12S Globulin Gel
4	0.2352a	---
5	0.1247b	0.1695a
6	0.09431c	---
7	0.08703cd	0.1405b
8	0.08068d	---
9	0.08095d	0.1053c

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

c. Effect of phytic acid concentration on the storage modulus (G') Due to the ability of phytic acid to bind directly to proteins at pH values below the isoelectric point, or through mulitvalent cation bridges at pH values above the isoelectric point, the properties (e.g. net charge) of the molecule surface are changed, as a result, it would be expected that phytic acid should play a role in gelation. As shown in Table 12, the G' for BSA gels formed in 5% phytic acid was significantly greater than those at lower phytic acid levels. In 3% phytic acid, however, the BSA gels had the smallest G' values and they were not significantly different from the gels formed in 1% phytic acid. In the case of the canola 12S globulin, the gels in 2% phytic acid had significantly greater G' values than the gels with no added phytic acid. Further addition of phytic acid did not seem to increase the rigidity of the canola gel; in fact, the gels formed in 5% phytic acid

were not significantly different from the one in 0% phytic acid.

Table 12: Effect of Phytic Acid Concentration on the Storage Modulus (G') of BSA and Canola 12S Globulin Gels

	G'	
% Phytic Acid	BSA Gel	12S Globulin Gel
0	---	50.54b
1	12420bc	---
2	13020b	72.71a
3	11670c	---
5	13900a	60.35ab

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

d. Effect of phytic acid concentration on the tan delta In Table 13, the results of statistical analyses indicate that the addition of phytic acid could significantly decrease the tan delta value or increase the elasticity of BSA gels yet has no effect on the 12S globulin gels (Appendices C and F). The most elastic BSA gels were formed in 5% phytic acid. There was no significant difference in the elasticity of the gels formed in 2 and 3% phytic acid.

Table 13: Effect of Phytic Acid Concentration on the tan Delta of BSA and Canola 12S Globulin Gels

	tan Delta	
% Phytic Acid	BSA Gel	12S Globulin Gel
0	---	0.1392a
1	0.1266a	---
2	0.1112b	0.1423a
3	0.1094b	---
5	0.09027c	0.1338a

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

e. Effect of calcium ion on the storage modulus (G') Certain amounts of calcium ions have been found to increase gel strength by forming calcium bridges (Mulvihill and Kinsella, 1988; Matsudomi et al., 1991). In addition, it has been reported that the calcium ion can bind to protein with phytic acid to form a ternary complex at pH values above the isoelectric point (Saio et al., 1968; Cheryan, 1980; Prattly et al., 1982; Grynspan and Cheryan, 1989). The presence of calcium ions, therefore, could significantly affect gel structures. The storage modulus of BSA gels as indicated in Table 14 and Appendices B and E, decreased in the presence of calcium but the G' of the canola gels was unaffected. The concentration of calcium ion did not appear to affect the rigidity of BSA gels as the G' values at 0.005 and 0.01M calcium were not significantly different. The reduction of the G' in the presence of calcium may due to excessive

aggregation (through direct calcium binding or ternary complex formation) causing the gel network to collapse.

Table 14: Effect of Calcium on the Storage Modulus (G') of BSA and Canola 12S Globulin Gels

	G'	
Molar of Ca^{2+} ion	BSA Gel	12S Globulin Gel
0	13880a	56.46a
0.005	12440b	---
0.01	11880b	65.94a

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

f. Effect of calcium ion on the tan delta As indicated in Table 15, the calcium ion plays a role in the elasticity of the BSA gel (Appendix C). The tan delta of the gels were significantly higher in the presence of calcium. In 0.005M calcium ion, the BSA gels had the highest tan delta values and those formed in 0.01M calcium ion had the second highest values. The tan delta of the globulin gels was not affected by the calcium ions (Appendix F).

Table 15: Effect of Calcium on the tan Delta of BSA and Canola 12S Globulin Gels

	tan Delta	
Molar of Ca^{2+} ion	BSA Gel	12S Globulin Gel
0	0.1009c	0.1455a
0.005	0.1180a	---
0.01	0.1114b	0.1314a

Column values followed by the same are not significantly different ($P \leq 0.10$)

2. Effects of Interactions of Experimental Factors on the Storage Modulus and Tan Delta

a. Interaction between pH and phytic acid concentration There were significant interactions between pH and phytic acid concentration in terms of the storage modulus and the tan delta values for the BSA gel (Appendix B and C). The experiment was not conducted in 5% phytic acid at pH 4 (zero value assumed in Fig. 14) since the protein precipitated from the solution during sample preparation. The effect of the interaction between pH and phytic acid concentration on the G' of the BSA gels is shown in Fig. 14. At pH 4, the values of G' in all concentrations of phytic acid were the lowest whereas the values at pH 5 were the highest. For all concentrations of phytic acid, the storage modulus gradually decreased as pH increased from 5 to 9. These observations corresponded to the trend seen for the main effect of pH. However, the mean values of G' within a pH level for different concentrations of phytic acid did not have a similar trend as observed for the main effect of phytic acid. It would appear that the high G'

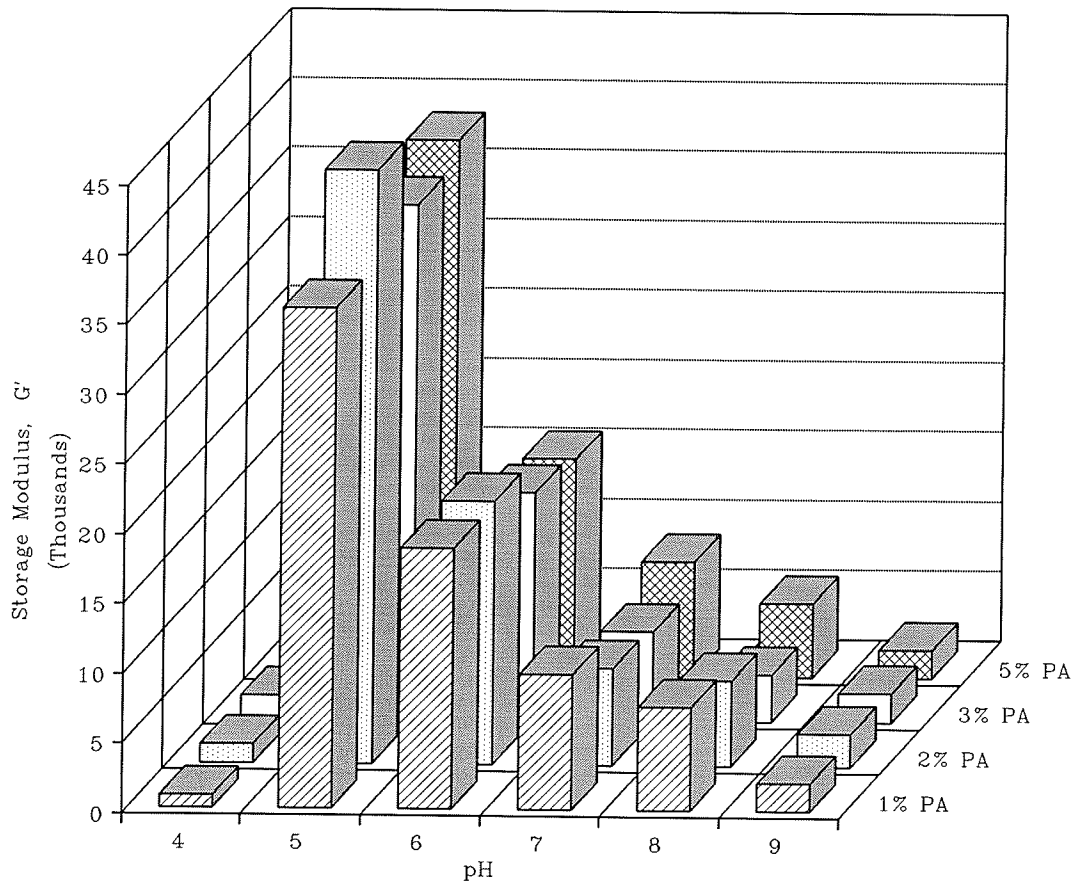


Figure 14: Effect of the Interaction between pH and Phytic Acid (PA) on G' Values of BSA Gels

value noted in 5% phytic acid was influenced by the behavior at pH 5 and 7. The fact that 2% phytic acid gave higher G' values than 1 and 3% phytic acid reflects the behavior at pH 5. For most other conditions, phytic acid had no apparent effect on the storage modulus.

In Fig. 15, the effect of the pH/phytic acid interaction on the tan delta value of the BSA gel is shown. The highest values of tan delta were obtained at pH 4 for all concentrations of phytic acid. At pH 5, the second highest values were observed. However, there was only a slightly decrease in the mean values of tan delta as pH value increased from 5 to 9; and, the variations of means between different concentrations of phytic acid were small and had no distinct order within a pH level. These observations are only corresponding to the main effect of pH but not to the effect of phytic acid. The main effect of phytic acid seemed to reflect the behavior at pH 4 only, where 1% phytic acid gave a significantly higher tan delta value.

There was no significant effect of the interaction between pH and phytic acid concentration on the storage modulus and the tan delta of the canola globulin gels (Appendix E and F).

b. Interaction between phytic acid and calcium concentrations The interaction between phytic acid and calcium concentrations had no significant effect on the storage modulus and the tan delta values for both BSA and the canola globulin gels (Appendices B, C, E, and F).

c. Interaction between pH and calcium Based on the results of statistical analyses, the effects of the interaction between pH and calcium on the G' and the tan

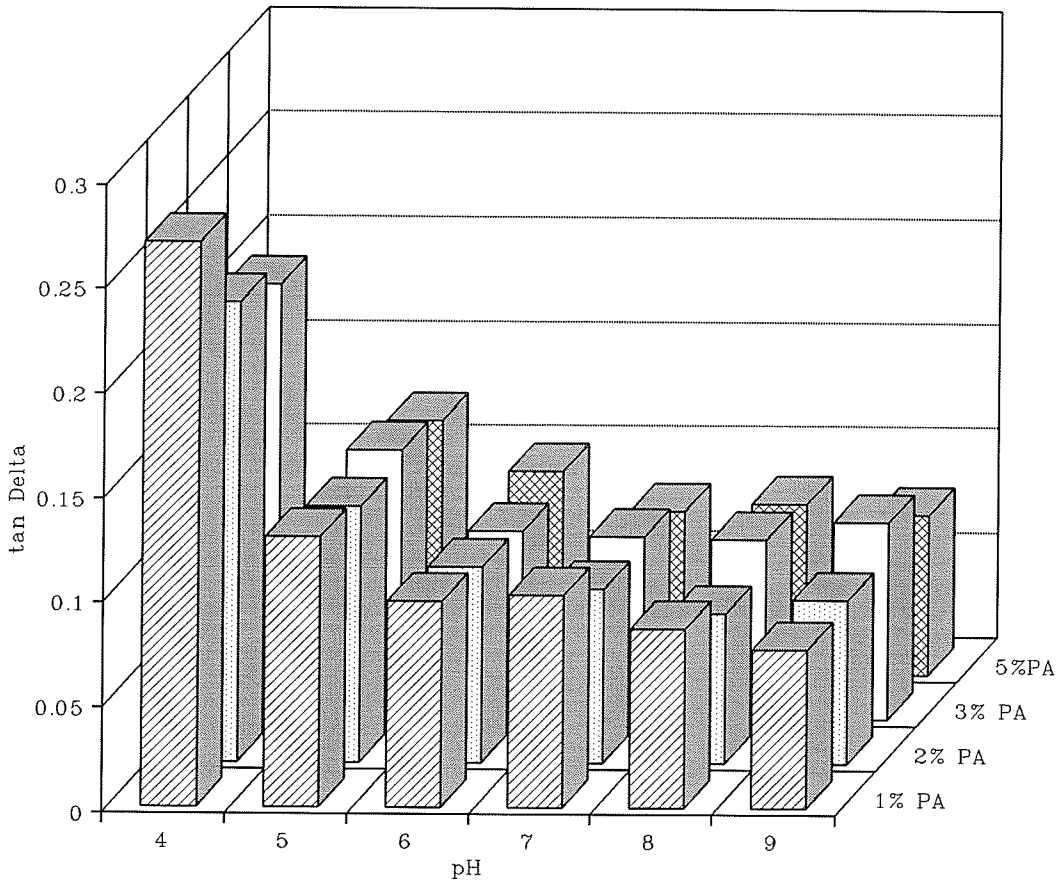


Figure 15: Effect of the Interaction between pH and Phytic Acid (PA) on tan Delta Values of BSA Gels

delta values of BSA gel are significant and shown in Fig. 16 and 17 (Appendix B and C).

In Fig. 16, the G' values at pH 5 were the highest regardless of the calcium ion concentration. As pH value increased from 5 to 9, the mean values of G' decreased. This follows the main effect of pH. At pH 4, 5 and 6, the gels formed in the presence of calcium had higher G' values but the gels prepared at pH 7, 8 and 9, were superior in the absence of the calcium ion. It would appear that gels formed at pH 5 and 6 allowed sufficient interaction between proteins to give a rigid structure. The presence of calcium at these pH values maintained or enhanced these interactions. At higher pH values, any interactions between calcium and BSA (either as a direct interaction or as part of a ternary complex) interfered with the interactions between protein molecules required for network formation. This was particularly noticeable at pH 9. The main effect seen for the calcium ion is indicative of the behaviour at pH 7 and above. Evaluation of the main effects indicated higher tan delta values were obtained with 0.005M calcium; this seemed to hold true at pH values between 5 and 8, as shown in Fig. 17. The presence of this low level of calcium apparently interfered with the development of elasticity in the network. Although the addition of more calcium did improve network structure slightly in this pH range, the tan delta values did not return to the low values observed in the absence of calcium. At pH 4, the presence of calcium resulted in a lowering of tan delta values. This is related to increased protein interactions responsible for the increased G' values. At pH 9, increasing calcium concentration resulted in continued deterioration of network elasticity. The delicate balance of attractive and repulsive forces necessary for good elasticity at this pH level (e.g. low tan delta in 0M calcium) was very sensitive to

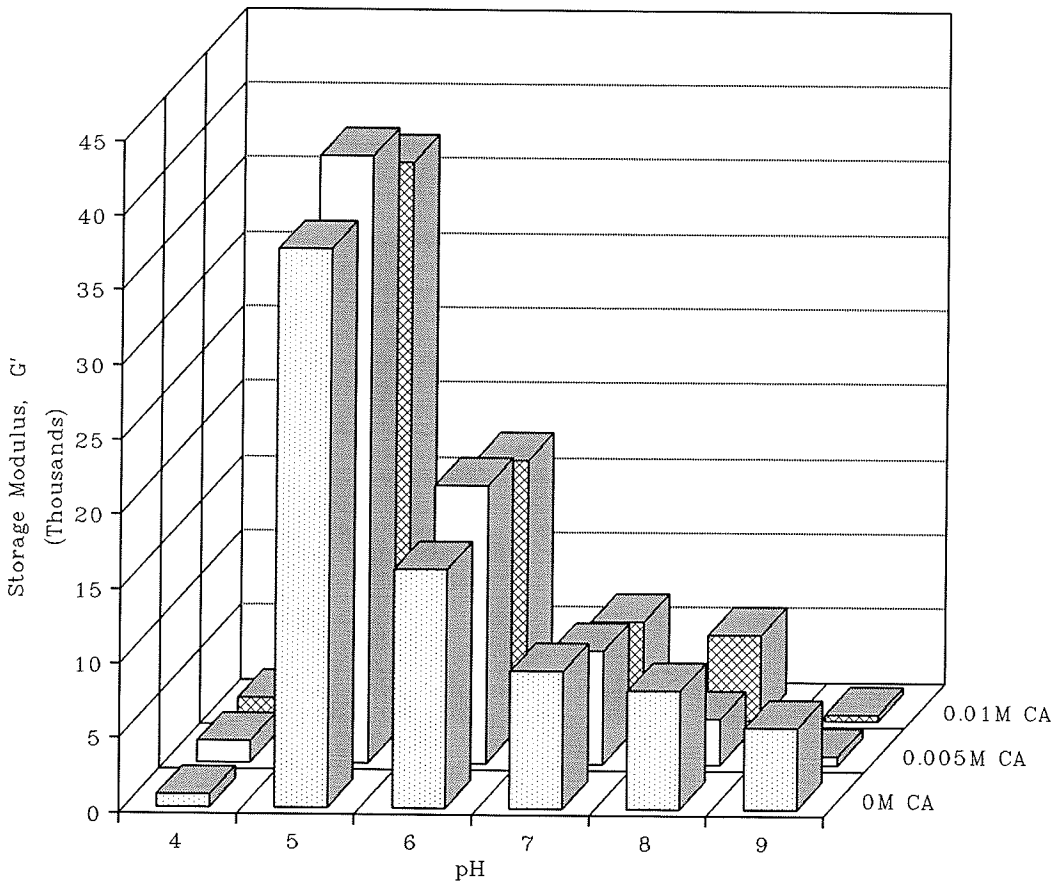


Figure 16: Effect of the Interaction between pH and Calcium (CA) on G' Values of BSA Gels

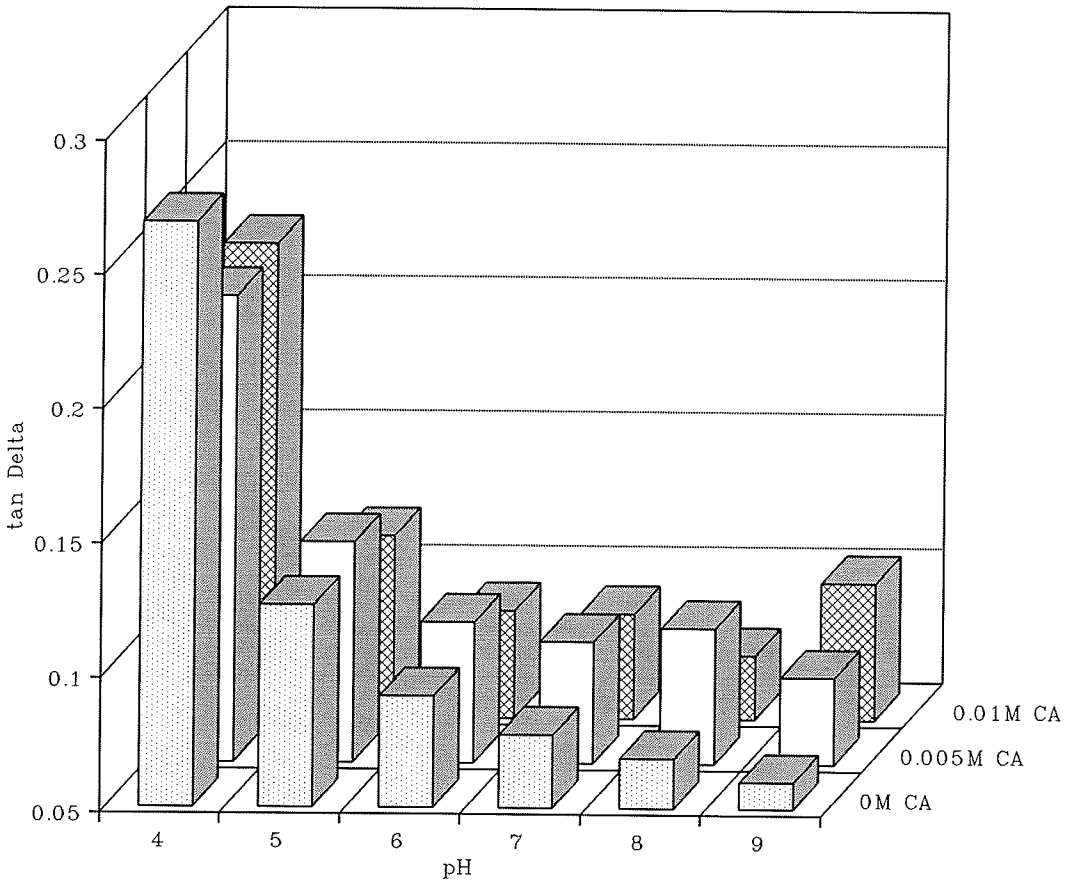


Figure 17: Effect of the Interaction between pH and Calcium (CA) on tan Delta Values of BSA Gels

interactions produced by the presence of calcium.

Although the interaction between pH and calcium had no significant effect on the storage modulus of the 12S globulin gel, it significantly affected the tan delta of the gel as shown in Fig. 18 (Appendices E and F). At pH 7 and 9, the canola gels formed in the absence of calcium always had higher tan delta values. At these pH values, interactions between protein molecules in the presence of calcium gave a more elastic network. At pH 5, the calcium ion seemed to reduce the elasticity of the canola gel presumably due to increased aggregation as interactions between protein molecules in the presence of calcium shifted the attractive repulsive balance too far to the attractive side. As indicated by the main effect of pH, the tan delta value did decrease when the pH value increased and the same trend was apparent in this 2-way interaction. The differences of the mean values between the gels formed in the absence and presence of calcium are also significant in terms of the interaction. The fact that there was no significant main effect of calcium ion observed previously is due to the opposite effects of the calcium at the different pH levels.

d. Interaction among all experimental factors In this section, only the effect of the 3-ways interaction on the storage modulus of BSA gel, as shown in Fig. 19, is discussed since there is no other significant 3-way interactions with significant effects (Appendices B, C, E and F). All the simple effects of pH, and concentrations of phytic acid and calcium in different treatments for the proteins gels are shown in Appendix B1 to B10, C1 to C10, E1 to E3 and F1 to F3.

The 3-way interaction on storage modulus for the BSA gels is shown in Fig. 19.

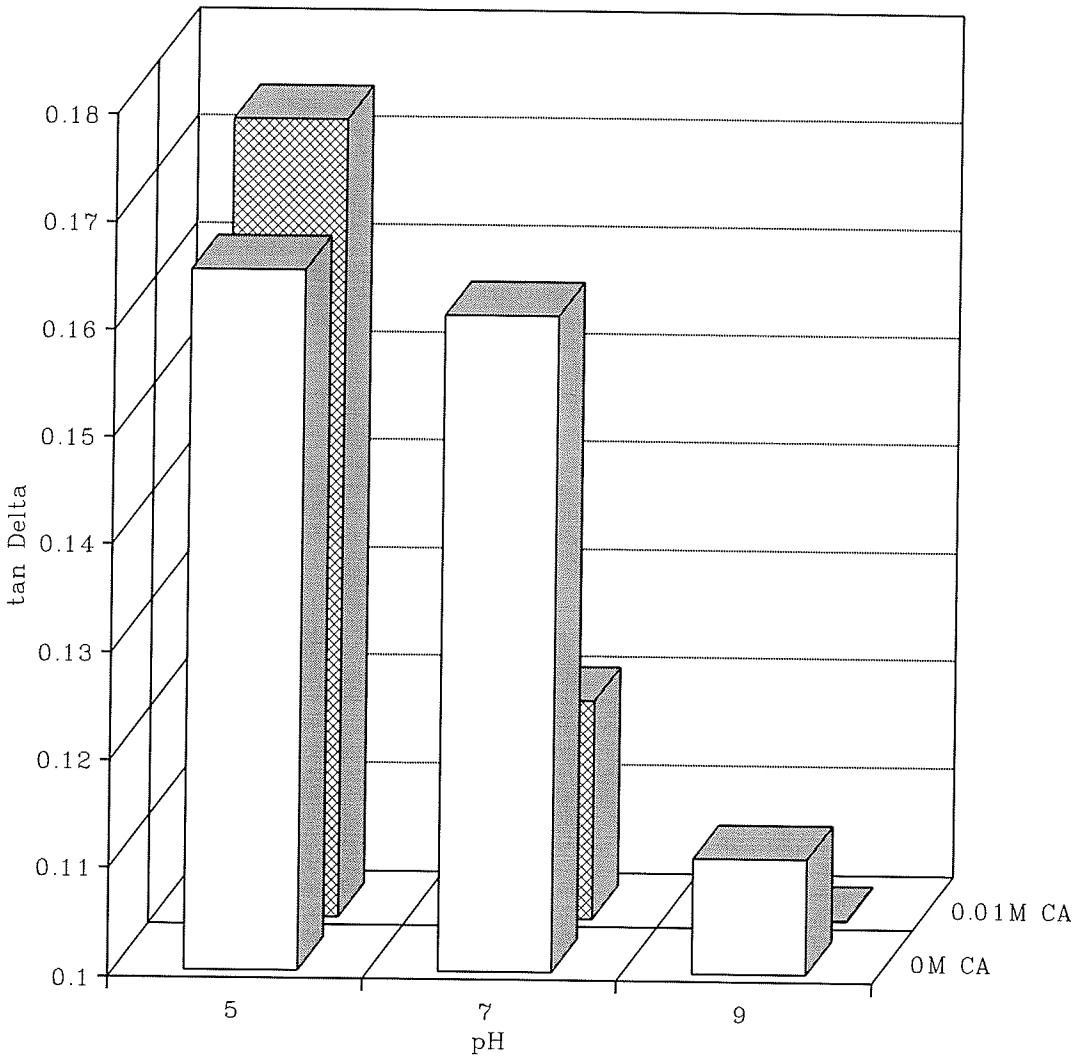


Figure 18: Effect of the Interaction between pH and Calcium (CA) on tan Delta Values of Canola 12S Globulin

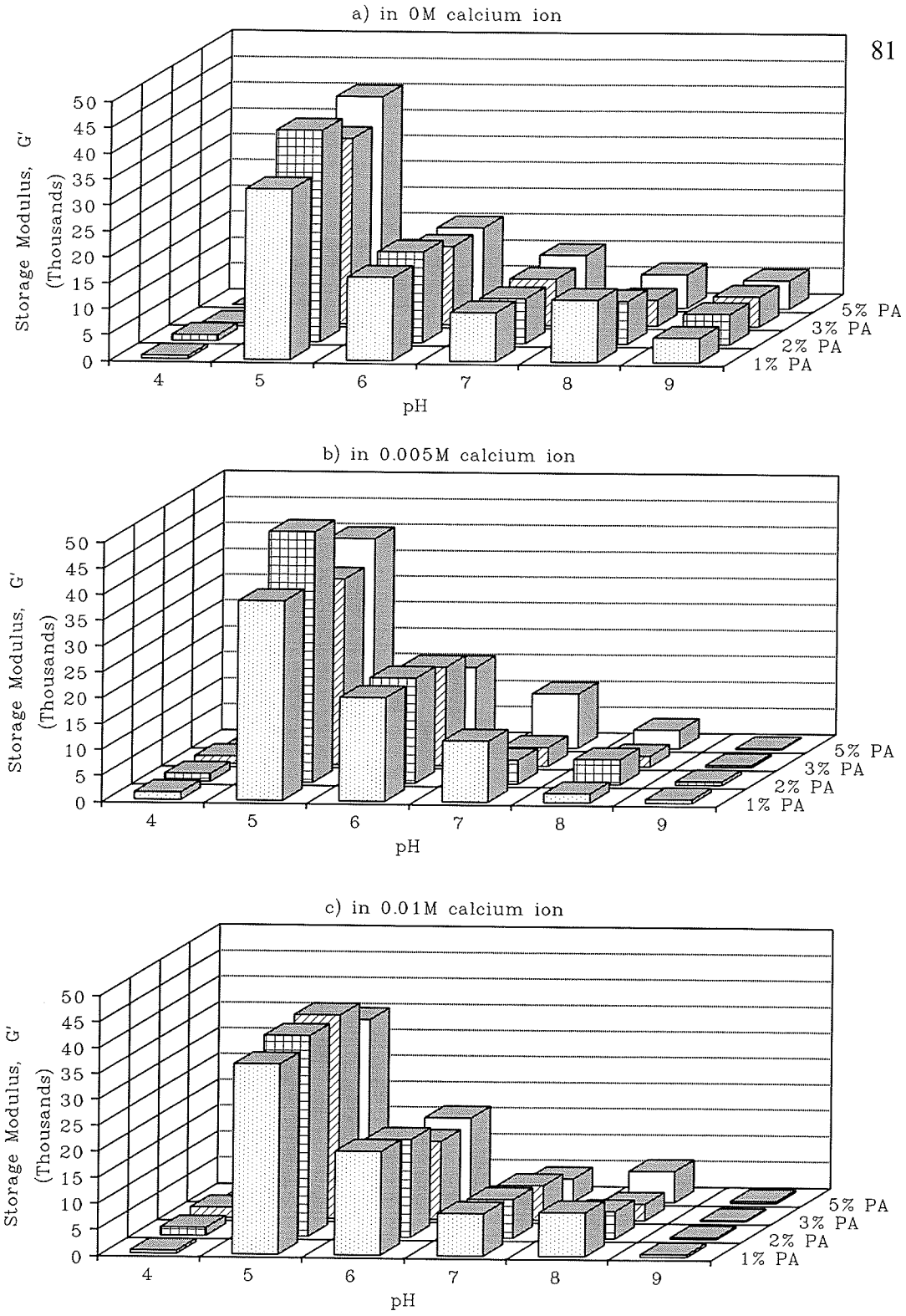


Figure 19: Effect of pH on the G' Values of BSA Gels. (PA-Phytic Acid)
a) in 0M, b) in 0.005M and c) in 0.01M calcium ion

No data was obtained at pH 4 with 5% phytic acid (all calcium levels) and 3% phytic acid (without calcium ion) because the protein precipitated out from the solution prior to heating. In Appendices B1 to B10, the effects of each factor on the storage moduli of BSA gels in all experimental treatments are shown. For all three concentrations of calcium ion, there were similar trends for the storage modulus of BSA gel: relatively small G' values were always observed at pH 4 and 9; the highest values were at pH 5; and, the G' decreased as pH value increased from 5 to 9. This corresponds to the trend seen for the main effect of pH. These results were similar to trends reported in the literature although the pH associated with maximum firmness differed. In a study of BSA gels, Matsudomi et al. (1991) found gels to have maximum hardness at pH 6.5. With increasing pH, the gel strength decreased and at pH values below 6.0, the gel hardness also markedly decreased. At pH 4, this pH effect was dominant as G' values were low regardless of phytic acid and calcium addition. In both 0 and 0.01M calcium, the 1% phytic acid samples produced gels with lower G' values than at higher phytic acid levels, but all gels were weak. At pH 5, the higher G' value associated with the 0.005M calcium level was significant only with the sample containing 2% phytic acid. Elastic and completely clear BSA gels have been reported in the presence of calcium chloride concentration up to 5mM (or 0.005M); above 10mM (or 0.01 M), the gels became turbid and weak (Matsudomi et al., 1991). This difference was primarily responsible for the 2-way interaction noted between pH and calcium ion. Variations due to phytate level at this pH were minimal; only the 2% phytic acid in 0.005M calcium had significantly higher G' values than gels made with other levels of phytic acid at this salt level. At pH 6,

addition of phytic acid had no effect on G' regardless of the calcium level; however, the presence of calcium resulted in higher G' values for the samples containing 1 and 2% phytic acid. These results concur with those seen for the 2-way interactions. At pH 7, phytic acid concentration had no effect on G' in either 0M or 0.01M calcium; this is also what was observed for the 2-way interaction of pH and phytic acid. With 0.005M calcium, however, G' values at 1% and 5% phytic acid were higher than those at 2 and 3%. Similarly at pH 8, the relationship between pH and phytic acid in 0.005M calcium is different from those in 0M and 0.01M calcium. It would appear that 0.005M calcium is a critical concentration for assessing the impact of phytic acid on protein gelation. The gels formed at pH 9 in the absence of calcium ions had more rigid network structures than the gels with calcium ions as was seen for the main effect of calcium and the 2-way interaction of calcium and pH. All pH 9 gels containing calcium produced very weak gels.

The formation of rigid network structures as measured by the storage modulus, of the BSA gels was found to be dependent on pH and the concentrations of the phytic acid and calcium ion. There was significant 3-way interaction among these parameters. In spite of this finding, pH clearly had the greatest impact on the rigidity of the BSA gels, although the responses of the rigidity at different levels of phytic acid and calcium ions often varied even at the same pH value. The elasticity of the gel, reflected by the tan delta value, was also dependent on pH and the concentrations of phytic acid and calcium ion. However, there was no interaction between phytic acid and calcium ion and no 3-way interaction (Appendices B and C). The effects of pH and concentrations of phytic

acid and the calcium ion on the tan delta value (or elasticity) of the BSA gels in all experimental treatments are shown in Appendix C1 to C10. Again, pH seemed to be the dominant factor in terms of elasticity of the BSA gels. High tan delta values, or inelastic gels, were observed at pH 4 in all experimental conditions but the elasticity of the gels increased significantly at pH 5. Protein aggregation at pH 4 would account for the low elasticity values. At pH 5 and above, the effect of pH was dependent on the presence of calcium. If no calcium was present the tan delta values gradually declined indicative of a more elastic network as would be expected as the net charge on the protein increased allowing for increased protein - solvent interaction while retaining a solid (although less rigid) structure. The inclusion of calcium under these conditions counteracts this improvement so that increases in pH from 6 to 9 do not give the same improvement in gel elasticity. The influence of phytic acid on BSA gels seems to be limited to gels formed at pH 4, and the differences observed at other pH values are minimal.

For the canola 12S globulin gels, the rigidity was determined by the pH value and phytic acid independently. The influence of calcium was though a 2-way interaction with pH. The increase in net protein charge with increased pH allows a more open elastic structure to develop. The G' value at pH 5 was due to the interactions within aggregates, whereas the high G' value at pH 9 reflected the increased rigidity of the network. As the level of pH increased, the gels became more elastic. At pH 7 and 9, the gels formed in the presence of the calcium ion were more elastic suggesting calcium was in some way contributing to crosslinking in the network. However, at pH 5, the gel with calcium ions was less elastic than the one without calcium ions because it promoted aggregation.

For both proteins gels, there was no significant 2-way interaction between the calcium ion and phytic acid. However for BSA this was a result of the counteracting responses at different pH values - as evidenced by the 3-way interaction.

C. The Effect Of Phytic Acid Binding On Thermal Gelation

In the following section, the effect of phytic acid binding to protein was assessed with regard to the thermal gelation of BSA and the canola 12S globulin. Since it is pH-dependent and different mechanisms (direct binding at pH range below the protein's isoelectric point and ternary complexation involving the multivalent cation bridge at the pH values above the isoelectric point) are currently believed to take place during binding, the effect on the thermal gelation is discussed in relation to the pH ranges below and above the isoelectric points of BSA and the canola globulin. In addition, the observed rheology of the gels in this experiment must take into consideration a combination of the phytic acid binding effect and the role of the physicochemical environments.

1. Bovine Serum Albumin

a. Below isoelectric point. Since BSA has its isoelectric point at pH 4.7, the binding effect only at pH 4 is discussed in this section. The binding at pH 4 was always greater than at other pH values in all concentrations of phytic acid and calcium. At this pH, the protein has a net positive charge and binds to phytic acid directly through electrostatic interaction. Increasing the level of phytic acid resulted in increased protein

binding but the presence of calcium did not have any apparent effect. Protein-protein interactions under this condition were enormous and hence extensive aggregation occurred. Despite the net positive charge, there are still a large number of negatively charged residues, as the pH is relatively close to the isoelectric point. The addition of phytic acid or calcium at this pH value resulted in slight improvement in network structure as the tan delta values were slightly lower and in some conditions the G' values were higher. It would appear that these compounds provide a bridge within aggregates by linking positively charged residues (phytic acid) or negatively charged residues (calcium). Based on the limited improvement in gel structure (not even close to what is obtained at pH value above the isoelectric point), this role is minor. The weakest and most inelastic gels were formed at this pH regardless of the concentrations of phytic acid and calcium ions.

b. Above isoelectric point Based on the phytic acid binding studies, there was no evidence to support the formation of the ternary complex (phytic acid-calcium-protein). The presence of calcium, in fact, discouraged the binding of phytic acid to BSA. Furthermore, the binding activity at pH values (5 - 9) above the BSA's isoelectric point was low even when calcium was not present.

At pH 5, the binding of phytic acid to BSA was relatively higher than the binding at other pH values since the protein molecules had slightly more positively charged sites than at higher pH values though the net charge was still negative. It was because the repulsion between the negatively charged sites and the negatively charged phytic acid molecules was not strong enough to prevent some contact and binding. The gels formed

at this pH had the greatest rigidity (highest G' values) but low elasticity. The proximity of this pH to the isoelectric point of BSA would again promote the formation of aggregates, however unlike the situation at pH 4, the aggregates at this pH had sufficient internal rigidity (high G' values) as has been reported previously (Gill and Tung, 1978). The fact that tan delta values were also relatively high provided evidence that there was aggregation rather than network formation. The inclusion of increasing concentrations of phytic acid at this pH resulted in increased binding and a slight increase in G' (Fig. 14) but no change in the tan delta values (Fig. 15). The phytic acid was probably acting to link positively charged sites on the proteins, thereby increasing rigidity without establishing a network. The addition of calcium resulted in decreased phytic acid binding, presumably due to competition between the protein and calcium for the phytic acid. Nevertheless, the presence of calcium also resulted in higher G' and tan delta values (Fig. 17). It would appear that the role of calcium in terms of protein gelation at this pH was independent of the phytic acid. The calcium promoted and increased aggregation, which was detrimental to network development, by linking negatively charged groups on the protein.

From pH 6 to 9, the binding was minimal and erratic. The rheology of gels formed under these experimental conditions was affected predominantly by pH and calcium. In general, the rigidity decreased but elasticity increased as pH values increased. This reflects a transition from an aggregated system to a more flexible three dimensional network. The presence of calcium decreased both the rigidity and elasticity (except at pH 6, where there were still aggregates contributing to the G' value) of the gels, especially

at pH 9. At these higher pH values, the interactions between calcium and the protein could disrupt the network or promote areas of aggregation. These small areas of aggregates did not contribute to the rigidity of the system. Thus the deterioration in network integrity in the presence of calcium appears to be the direct effect of calcium rather than through a modification of the relationship between phytic acid and protein. The minimal binding of phytic acid to BSA itself appeared to have little effect on the thermal gelation.

2. Canola 12S globulin

a. Below isoelectric point The canola globulin has an isoelectric point of 7.25; as a result, the following discussion includes the phytic acid binding at pH values 5 and 7.

Higher binding was observed at pH 5 in all concentrations of phytic acid and calcium as would be expected as the net positive charge is greater at pH 5. At this pH, increased amounts of phytic acid resulted in increased binding but the presence of calcium decreased the binding of phytic acid to the canola globulin (Appendix D2 and D3). The rheology of the gels formed at pH 5 and 7 shared similar characteristics, weak and inelastic (Appendices E1 to E3 and F1 to F3). The phytic acid binding to the canola globulin played a very minor role in the thermal gelation in that a low level of phytic acid (2%) increased the G' values at pH 5, but this increase was reversed as a higher concentration of phytic acid was used (Appendices E2 and F2). The increase in interactions between protein molecules due to increased phytic acid binding led to

increased aggregation and increased rigidity in the system, as was seen with BSA. As the concentration of phytic acid was increased further, the nature of the interaction seemed to disrupt rather than contribute to the structures present.

Despite the fact that increased calcium resulted in decreased phytic acid binding, as was the case with BSA, this had no impact on network characteristics. The degree of competition under these conditions was insufficient to have an impact on the gelling properties.

b. Above isoelectric point The binding of phytic acid at pH 9 was low and became even lower as phytic acid concentration was increased or calcium was added. However, the gels formed at this pH value were the most rigid and elastic. As indicated in Appendices E2 and F2, there were no significant differences in term of rigidity and elasticity for the gels formed upon the addition of phytic acid or calcium. This was not unexpected because of the low binding at this pH level. Clearly for the 12S canola globulin, pH is the key factor in controlling the characteristics of thermally induced gels.

V. CONCLUSIONS AND RECOMMENDATIONS

A. Bovine Serum Albumin

1. Phytic Acid Binding

Phytic acid binding to bovine serum albumin (BSA) was found to be a function of pH and the concentrations of phytic acid and calcium and their effects on the binding were dependent. In addition, pH was the most influential factor.

The extent of phytic acid binding to BSA was the highest at pH 4 in all concentrations of phytic acid (1, 2, 3 and 5%) and the calcium ion (0.005 and 0.01M). With an increased amount of phytic acid, binding increased. The presence of calcium was found to have no impact on the binding. This indicated that the mechanism was a direct electrostatic binding between phytic acid and BSA. Moreover, the binding to BSA was not satisfied even at the 5% phytic acid level.

Regardless of the concentrations of phytic acid and calcium, the second highest binding was always observed at pH 5. The binding mechanism was a direct electrostatic interaction between BSA and phytic acid. With no calcium, the extent of binding at this pH increased as the amount of phytic acid increased. Nevertheless, binding was maximized in 3% phytic acid level in the presence of calcium. The reduction of phytic acid binding in the presence of calcium was due to the competition between protein and calcium for binding sites on the phytic acid.

At pH 6 and above, binding to BSA was minimal and erratic. There was a lack

of evidence for the formation of a ternary complex. The presence of calcium diminished the extent of binding further due to the competition between calcium and protein for phytic acid.

2. Gel Rheological Studies

The formation of a network structure of the BSA gels was found to depend on pH and the concentrations of the phytic acid and calcium. Their effects were dependent. Despite that, pH clearly had the greatest impact on the rigidity of the BSA gels. The elasticity of the gels was also found to be a function of pH and the concentrations of phytic acid and calcium but only the interaction between pH and calcium was significant.

a. Gel rigidity There was a similar trend in terms of the effect of pH on the rigidity of BSA gel regardless of the concentrations of phytic acid and calcium. The gels formed at pH 4 and 9 were weaker but the gels formed at pH 5 were the strongest; and, the rigidity declined as pH values increased from 5 to 9.

Phytic acid had a limited effect on the rigidity at most levels of pH. However, there is a critical concentration of calcium, 0.005M, for assessing the impact of phytic acid on the rigidity of gels formed at pH 5, 7 and 8. At pH 5, significantly rigid gels were formed in 2% phytic acid. At pH 7 and 8, rigid gels were formed in 1 and 5% phytic acid.

At pH 6, the concentration of calcium (0.005M and 0.01M) had no impact on the effect of phytic acid but its presence increased the rigidity in 1 and 2% phytic acid. On the other hand, the gels formed at pH 9 in the presence of calcium had weaker network

structures than the gels without calcium.

b. Gel elasticity Inelastic gels were formed at pH 4 but they improved significantly at pH 5 in all experimental conditions. With no calcium, the elasticity increased while retaining a reasonably rigid structure as the pH level raised. There was no such improvement in elasticity in the presence of calcium. The influence of phytic acid on BSA gels was limited at all pH levels.

3. Effect of Phytic Acid Binding on Thermal Gelation

At pH 4, the gels in all concentrations of phytic acid and calcium had extremely weak and inelastic structures since there were extensive protein aggregations, due to the proximity of the isoelectric point and phytic acid binding to BSA. Slight improvement in network structures were accomplished by adding phytic acid or calcium. They provided bridges within aggregates by linking the positively and negatively charged residues. However, their roles were minor.

Regardless of the concentrations of phytic acid and calcium, the gels formed at pH 5 had the greatest rigidity but low elasticity since the pH level was close to the isoelectric point and the extent of phytic acid binding to BSA was great. The protein molecules were linked by phytic acid and calcium. Although both compounds increased the strength of localized aggregates, they promoted aggregation instead of three dimensional network structure.

From pH 6 to 9, the minimal phytic acid binding to BSA had very limited impact on the thermal gelation. The rigidity and elasticity of the gels were influenced

predominantly by pH and calcium.

B. Canola 12S Globulin

1. Phytic Acid Binding

The degree of binding to the canola globulin was affected by pH and the concentrations of phytic acid and calcium with significant interactions.

At pH 5 and 7, binding increased as the level of phytic acid (2 and 5%) increased but decreased as the pH value increased and/or in the presence of the calcium ion (in 0.01M). The highest binding was achieved at pH 5 in all experimental treatments. Phytic acid was found to bind to the canola globulin electrostatically.

At pH 9, phytic acid binding was minimal. Again, there was a lack of formation of the ternary complex. The effect of phytic acid concentration on the binding of phytic acid to the canola globulin depended on the calcium ion concentration. In 5% phytic acid, the extent of binding in the absence of calcium was higher than the binding in 2% phytic acid while the reverse was true in the presence of calcium. The presence of calcium again discouraged the binding to the canola globulin.

2. Gel Rheological Studies

For the canola 12S globulin gels, the rigidity was determined by pH and phytic acid independently. The pH and calcium were found to affect the elasticity with significant interaction. Similar to BSA gels, the most effective factor was found to be the pH level.

a. Gel rigidity The addition of phytic acid produced more rigid gel structures in most treatments. At pH 5, the gels had certain rigidity which was due to the closeness of lumpy aggregates. The weakest gel structures were formed at pH 7, which is almost at the isoelectric point. The gels formed at pH 9 were the most rigid regardless of the concentrations of phytic acid and calcium.

b. Gel elasticity The most elastic gels were formed at pH 9 yet the least elastic at pH 5. In 0.01M calcium, the gels formed at pH 7 and 9 were more elastic. The calium ion in this concentration, however, did not have the same effect on the elasticity of the gels formed at pH 5.

3. Effect of Phytic Acid Binding on Thermal Gelation

The gels formed at pH 5 and 7 in any concentration of phytic acid and calcium were weak and inelastic. The binding of phytic acid to the canola globulin had a very minor role in the thermal gelation. The increase in the binding only led to increased rigidity of the localized aggregates, as was seen with BSA. The elasticity of the canola globulin gels was not affected by the binding. Furthermore, the decreased phytic acid binding, due to the addition of calcium (in 0.01M) had no impact on the characteristics of the gels.

At pH 9, the binding of phytic acid was minimal and had no significant effect on the rheology of the gels regardless of the concentrations of phytic acid and calcium.

C. Recommendations

In order to help the prediction of phytic acid binding to protein and its effect on thermal gelation (or any functional property), the quaternary structure and the characteristic of the molecule surface (eg. charge distribution) of the protein must be determined. Further study into the affinity of the protein and multivalent cation(s) to phytic acid should also provide more insight. In addition, the distinction between insoluble calcium phytate and ternary complex must be made in future solubility and binding studies.

To utilize the canola isolate as a gelling agent in food products, the rheological properties of gels formed at low pH values (below and near its isoelectric point) must be improved since most foods have their pH below 7. The improvement might be done by chemical or enzymatic protein modification. Since the presence of phytic acid does not impair network formation at pH values higher than the isoelectric point, it should not be a concern in further protein isolation studies focusing on protein functionality.

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VII. APPENDICES

Appendix A: The Binding Extent of BSA with Phytic Acid (mole of phytic acid/mole of BSA)

Dependent Variable: Binding Extent

Source	DF	Sum of Squares	Mean Square	F Value	Pr. > F
Model	71	1479.65	20.8402	149.76	0.0001
Error	72	10.0193	0.139158		
Corrected Total	143	1489.67			

R-Square	C.V.	Root MSE	Binding Extent Mean
0.993274	25.9308	0.373038	1.43859

Source	DF	Type III SS	Mean Square	F Value	Pr. > F
pH	5	875.626	175.125	1258.47	0.0001
Phytic acid	3	2.95740	0.985800	7.08000	0.0003
pH*Phytic acid	15	306.414	20.4276	146.79	0.0001
Ca^{2+}	2	43.4535	21.7268	156.13	0.0001
pH*Ca^{2+}	10	105.992	10.5992	76.1700	0.0001
Phytic acid* Ca^{2+}	6	56.9391	9.48986	68.2000	0.0001
pH*Phytic acid*Ca^{2+}	30	88.2702	2.94234	21.1400	0.0001

Appendix A1 : Simple Effect of pH on Phytic Acid Binding to BSA in 0M Ca^{2+}

	Binding Extent			
pH	1% PA	2% PA	3% PA	5% PA
4	3.872±0.1110a	5.720±0.4192a	2.453±0.4312b	11.60±0.0852a
5	3.247±0.1531a	2.026±0.1118bc	5.427±0.0890a	5.897±0.1711b
6	1.294±0.4122b	0.9710±1.2290c	-1.644±0.4395f	0.7015±0.2432d
7	1.193±0.4168bc	1.381±0.0209c	1.113±0.0664c	4.056±0.2541c
8	0.8124±0.4447bc	1.668±0.5036bc	-0.7158±0.5073e	0.5647±0.2541d
9	0.4950±0.4772c	2.707±0.5589b	0.1200±0.2834d	-1.953±0.0867e

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix A2 : Simple Effect of pH on Phytic Acid Binding to BSA in 0.005M Ca^{2+}

	Binding Extent			
pH	1% PA	2% PA	3% PA	5% PA
4	3.146±0.0351a	5.574±0.1226a	9.001±0.2044a	10.27±0.5607a
5	2.221±0.2336b	1.846±0.3854b	4.853±0.2623b	1.156±0.7474b
6	-0.0164±0.4098c	-0.5517±0.4838c	0.8865±0.3119c	-0.4625±0.5621c
7	-0.7572±0.5410d	-1.610±0.0346d	-0.6105±0.1746d	-2.378±0.0933d
8	0.2585±0.1639c	-0.3209±0.0190c	-2.718±0.6929f	-0.8199±0.1513c
9	2.861±0.0756a	2.064±0.0882b	-1.604±0.1570e	-4.884±0.0509e

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix A3 : Simple Effect of pH on Phytic Acid Binding to BSA in 0.01M Ca^{2+}

	Binding Extent			
pH	1% PA	2% PA	3% PA	5% PA
4	3.130±0.1635a	5.748±0.1577a	8.753±0.1460a	9.811±0.2805a
5	0.3304±0.1285c	-2.007±0.3679f	2.809±0.4963b	-0.1980±0.4200b
6	-1.542±0.4564d	0.2163±0.0711d	0.3005±0.3401c	0.1815±0.9368b
7	-1.206±0.0000d	-1.573±0.2799e	-0.7200±0.5543d	-3.364±0.1400d
8	1.457±0.2458b	2.324±0.0351b	0.8259±.2333c	-1.320±0.2340c
9	0.6855±0.3970c	0.8300±0.1227c	-0.4955±0.2623d	-1.816±0.5607c

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

Appendix A4 : Simple Effect of Phytic Acid Concentration on the Binding of Phytic Acid to BSA in 0M Ca^{2+}

	Binding Extent					
% PA	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
1	3.872c	3.247c	1.294a	1.193b	0.8124ab	0.4950b
2	5.720b	2.026d	0.9710a	1.381b	1.668a	2.707a
3	2.453d	5.427b	-1.644b	1.113b	-0.7158c	0.1200b
5	11.60a	5.897a	0.7015a	4.056a	0.5647b	-1.953c

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A1

Appendix A5: Simple Effect of Phytic Acid Concentration on the Binding of Phytic Acid to BSA in 0.005M Ca^{2+}

	Binding Extent					
% PA	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
1	3.146d	2.221b	-0.01640ab	-0.7572a	0.2585a	2.861a
2	5.574c	1.846bc	-0.5517b	-1.610b	-0.3209ab	2.064b
3	9.001b	4.853a	0.8865a	-0.6105a	-2.718c	-1.604c
5	10.27a	1.156c	-0.4625b	-2.378c	-0.8199b	-4.884d

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A2

Appendix A6 : Simple Effect of Phytic Acid Concentration on the Binding of Phytic Acid to BSA in 0.01M Ca^{2+}

	Binding Extent					
% PA	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
1	3.130d	0.3304b	-1.542b	-1.206ab	1.457b	0.6855a
2	5.748c	-2.007c	0.2163a	-1.573b	2.324a	0.8300a
3	8.753b	2.809a	0.3005a	-0.7200a	0.8259c	-0.4955b
5	9.811a	-0.1980b	0.1815a	-3.364c	-1.320d	-1.816c

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A3

Appendix A7 : Simple Effect of Ca^{2+} on the Binding of Phytic Acid to BSA in 1% Phytic Acid

	Binding Extent					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	3.872a	3.247a	1.294a	1.193a	0.8124ab	0.4950b
0.005	3.146b	2.221b	-0.01640b	-0.7572b	0.2585b	2.861a
0.01	3.130b	0.3304c	-1.542c	-1.206b	1.457a	0.6855b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A1 to A3

Appendix A8 : Simple Effect of Ca^{2+} on the Binding of Phytic Acid to BSA in 2% Phytic Acid

	Binding Extent					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	5.720a	2.026a	0.9710a	1.381a	1.668a	2.707a
0.005	5.574a	1.846a	-0.5517a	-1.610b	-0.3209b	2.064a
0.01	5.748a	-2.007b	0.2163a	-1.573b	2.324a	0.830b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A1 to A3

Appendix A9 : Simple Effect of Ca^{2+} on the Binding of Phytic Acid to BSA in 3% Phytic Acid

	Binding Extent					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	2.453b	5.427a	-1.644b	1.113a	-0.7158b	0.1200a
0.005	9.001a	4.853a	0.8865a	-0.6105b	-2.718c	-1.604c
0.01	8.753a	2.809b	0.3005a	-0.7200b	0.8259a	-0.4955b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A1 to A3

Appendix A10 : Simple Effect of Ca^{2+} on the Binding of Phytic Acid to BSA in 5% Phytic Acid

	Binding Extent					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	11.60a	5.897a	0.7015a	4.056a	0.5647a	-1.953a
0.005	10.27b	1.156b	-0.4625a	-2.378b	-0.8199b	-4.884b
0.01	9.811b	-0.1980c	0.1815a	-3.364c	-1.320b	-1.816a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix A1 to A3

Appendix B: The Storage Modulus (G') of BSA

Dependent Variable: Storage Modulus, G'

Source	DF	Sum of Squares	Mean Square	F Value	Pr. > F
Model	67	2394647744	354710265	83.41	0.0001
Error	68	291381729	4285025		
Corrected Total	135	24237869473			

R-Square	C.V.	Root MSE	Storage Modulus Mean
0.987978	16.2802	2070.03	12715.1

Source	DF	Type III SS	Mean Square	F Value	Pr. > F
pH	5	23027368168	4605473634	1074.78	0.0001
Phytic acid	3	77555733	25851911	6.03	0.0011
pH*Phytic acid	14	221928781	15852056	3.7	0.0001
Ca^{2+}	2	52817256	26408628	6.16	0.0035
pH*Ca^{2+}	10	289431882	28943188	6.75	0.0001
Phytic acid* Ca^{2+}	6	33498860	5583143	1.30	0.2678
pH*Phytic acid*Ca^{2+}	27	243887066	9032854	2.11	0.0070

Appendix B1 : Simple Effect of pH on the G'of the BSA Gel in 0M Ca^{2+}

	G'			
pH	1% PA	2% PA	3% PA	5% PA
4	625.5±125.2d	1059±157.7d	---	---
5	32800±9050a	40800±2121a	35750±3182a	40250±1626a
6	16050±353.6b	17500±424.3b	15100±565.7b	15300±3253b
7	9375±1025bc	8665±2170c	8935±1082c	10005±275.8c
8	11930±3493bc	8235±289.9c	5025±601.0d	6380±2744c
9	4675±332.3cd	5850±2616c	5700±523.3d	5385±3061c

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix B2 : Simple Effect of pH on the G'of the BSA Gel in 0.005M Ca^{2+}

	G'			
pH	1% PA	2% PA	3% PA	5% PA
4	1395±417.2d	1520±396.0d	1365±445.5c	---
5	38350±70.71a	48400±2263a	35800±3818a	40100±1838a
6	20000±989.9b	20200±141.4b	18900±565.7b	15400±565.7b
7	11700±707.1c	4645±643.5c	3610±1400c	10450±353.6c
8	1715±91.92d	4775±671.8c	2005±615.2c	3625±1082d
9	788.0±164.0d	745.5±306.2d	381.0±69.30c	221.5±123.7e

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix B3 : Simple Effect of pH on the G'of the BSA Gel in 0.01M Ca^{2+}

	G'			
pH	1% PA	2% PA	3% PA	5% PA
4	597.0±230.5d	1520±254.6e	1890±226.3cd	---
5	36550±1485a	38600±707.1a	39150±353.6a	34800±2687a
6	19900±989.9b	18800±707.1b	14950±5303b	15900±2970b
7	8125±5904c	7400±565.7c	6535±2920c	4315±898.0cd
8	8440±1513c	5240±1867d	3070±961.7cd	5960±2616c
9	504.5±188.8d	324.0±90.51e	194.0±36.77d	374.0±72.12d

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

Appendix B4 : Simple Effect of Phytic Acid Concentration on the G' of the BSA Gel in 0M Ca^{2+}

	G'					
% PA	pH4	pH5	pH6	pH7	pH8	pH9
1	625.5b	32800a	16050a	9375a	11930a	4675a
2	1059a	40800a	17500a	8665a	8235ab	5850a
3	---	35750a	15100a	8935a	5025b	5700a
5	---	40250a	15300a	10005a	6380b	5385a

Column values followed by the same letter are not sginificantly different (P≤0.10)
Standard deviations are shown in Appendix B1

Appendix B5 : Simple Effect of Phytic Acid Concentration on the G' of the BSA Gel in 0.005M Ca^{2+}

	G'					
% PA	pH4	pH5	pH6	pH7	pH8	pH9
1	1395a	38350a	20000a	11700a	1715b	788.0a
2	1520a	48400b	20200a	4645b	4775a	745.5a
3	1365a	35800a	18900a	3610b	2005b	381.0ab
5	---	40100a	15400b	10450a	3625a	221.5b

Column values followed by the same letter are not sginificantly different (P≤0.10)
Standard deviations are shown in Appendix B2

Appendix B6 : Simple Effect of Phytic Acid Concentration on the G' of the BSA Gel in 0.01M Ca^{2+}

	G'					
% PA	pH4	pH5	pH6	pH7	pH8	pH9
1	597.0b	36550ab	19900a	8125a	8440a	504.5a
2	1520a	38600a	18800a	7400a	5240ab	324.0ab
3	1890a	39150a	14950a	6535a	3070b	194.0b
5	---	34800b	15900a	4315a	5960ab	374.0ab

Column values followed by the same letter are not sginificantly different (P≤0.10)
Standard deviations are shown in Appendix B3

Appendix B7: Simple Effect of Ca^{2+} on the G' of the BSA Gel in 1% Phytic Acid

	G'					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	625.5b	32800a	16050b	9375a	11930a	4675a
0.005	1395a	38350a	20000a	11700a	1715b	788.0b
0.01	597.0b	36550a	19900a	8125a	8440a	504.5b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix B1 to B3

Appendix B8: Simple Effect of Ca^{2+} on the G' of the BSA Gel in 2% Phytic Acid

	G'					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	1059a	40800b	17500c	8665a	8235a	5850a
0.005	1520a	48400a	20200a	4645b	4775b	745.5b
0.01	1520a	38600b	18800b	7400ab	5240b	324.0b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix B1 to B3

Appendix B9: Simple Effect of Ca^{2+} on the G' of the BSA Gel in 3% Phytic Acid

	G'					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	---	35750a	15100a	8935a	5025a	5700a
0.005	1365a	35800a	18900a	3610b	2005b	381.0b
0.01	1890a	39150a	14950a	6535ab	3070b	194.0b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix B1 to B3

Appendix B10: Simple Effect of Ca^{2+} on the G' of the BSA Gel in 5% Phytic Acid

	G'					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	---	40250a	15300a	10010a	6380a	5385a
0.005	---	40100a	15400a	10450a	3625a	221.5b
0.01	---	34800b	15900a	4315b	5960a	374.0b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix B1 to B3

Appendix C: The tan Delta of BSA Gel

Dependent Variable: tan Delta

Source	DF	Sum of Squares	Mean Square	F Value	Pr. > F
Model	67	0.363694	0.00542827	15.65	0.0001
Error	68	0.0235926	0.00034695		
Corrected Total	135	0.387287			

R-Square	C.V.	Root MSE	tan Delta Mean
0.939082	16.9021	0.0186266	0.110203

Source	DF	Type III SS	Mean Square	F Value	Pr. > F
pH	5	0.315602	0.0631203	181.93	0.0001
Phytic acid	3	0.00486831	0.00162277	4.68	0.0050
pH*Phytic acid	14	0.0116935	0.00083525	2.41	0.0085
Ca^{2+}	2	0.00372225	0.00186113	5.36	0.0069
pH*Ca^{2+}	10	0.0136769	0.00136769	3.94	0.0003
Phytic acid* Ca^{2+}	6	0.00271911	0.00045319	1.31	0.2664
pH*Phytic acid*Ca^{2+}	27	0.0114125	0.00042269	1.22	0.2530

Appendix C1: Simple Effect of pH on the tan Delta of BSA Gel in 0M Ca^{2+}

	tan Delta			
pH	1% PA	2% PA	3% PA	5% PA
4	0.304±0.0249a	0.230±0.0011a	---	---
5	0.124±0.0052b	0.136±0.0025b	0.115±0.0010a	0.124±0.0039a
6	0.0969±0.0013bc	0.101±0.0008c	0.0787±0.0064b	0.0871±0.0010b
7	0.0919±0.0023c	0.0738±0.0016d	0.0678±0.0040b	0.0746±0.0044c
8	0.0705±0.0024cd	0.0606±0.0055e	0.0674±0.0011b	0.0754±0.0041c
9	0.0583±0.0017d	0.0633±0.0030e	0.0497±0.0041c	0.0676±0.0049c

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix C2: Simple Effect of pH on the tan Delta of BSA Gel in 0.005M Ca^{2+}

	tan Delta			
pH	1% PA	2% PA	3% PA	5% PA
4	0.236±0.0683a	0.216±0.0022a	0.218±0.0015a	---
5	0.142±0.0002b	0.116±0.0001b	0.137±0.0001b	0.132±0.0002a
6	0.110±0.0014b	0.0926±0.0011c	0.105±0.0013c	0.101±0.0006b
7	0.100±0.0022b	0.0919±0.0003c	0.110±0.0009c	0.0782±0.0050c
8	0.112±0.0025b	0.0879±0.0069cd	0.120±0.0152cb	0.0819±0.0018c
9	0.0805±0.0028b	0.0817±0.0033d	0.0806±0.0023d	0.0861±0.0042c

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix C3: Simple Effect of pH on the tan Delta of BSA Gel in 0.01M Ca^{2+}

	tan Delta			
pH	1% PA	2% PA	3% PA	5% PA
4	0.269±0.0074a	0.213±0.0025a	0.196±0.0009a	---
5	0.121±0.0024b	0.113±0.0003b	0.131±0.0045abc	0.106±0.0009a
6	0.0884±0.0001bc	0.0865±0.0005bc	0.0838±0.0025bc	0.101±0.0062a
7	0.113±0.0052b	0.0827±0.0033bc	0.0815±0.0039bc	0.0791±0.0003bc
8	0.0739±0.0267c	0.0658±0.0235c	0.0690±0.0189c	0.0853±0.0017b
9	0.0884±0.0060bc	0.0893±0.0153bc	0.151±0.0618ab	0.0749±0.0038c

Column values followed by the same letter are not significantly different ($P \leq 0.10$)

Appendix C4: Simple Effect of Phytic Acid Concentration on the tan Delta of the BSA Gel in 0M Ca^{2+}

	tan Delta					
% PA	pH4	pH5	pH6	pH7	pH8	pH9
1	0.3042a	0.1243b	0.09690ab	0.09190a	0.07050ab	0.05825ab
2	0.2302b	0.1363a	0.1014a	0.07380b	0.06060b	0.06325a
3	---	0.1152b	0.07865c	0.06780b	0.06735ab	0.04970b
5	---	0.1241b	0.08715bc	0.07455b	0.07545a	0.06760a

Column values followed by the same letter are not siginificantly different (P≤0.10)
Standard deviations are shown in Appendix C1

Appendix C5: Simple Effect of Phytic Acid Concentration on the tan Delta of the BSA Gel in 0.005M Ca^{2+}

	tan Delta					
% PA	pH4	pH5	pH6	pH7	pH8	pH9
1	0.2363a	0.1420a	0.1097a	0.1004b	0.1115ab	0.08050a
2	0.2155a	0.1161d	0.09255c	0.09185c	0.08785bc	0.08165a
3	0.2176a	0.1365b	0.1045b	0.1102a	0.1193a	0.08055a
5	---	0.1321c	0.1013b	0.07815d	0.08185c	0.08610a

Column values followed by the same letter are not siginificantly different (P≤0.10)
Standard deviations are shown in Appendix C2

Appendix C6: Simple Effect of Phytic Acid Concentration on the tan Delta of the BSA Gel in 0.01M Ca^{2+}

	tan Delta					
% PA	pH4	pH5	pH6	pH7	pH8	pH9
1	0.2692a	0.1205b	0.08840b	0.1125a	0.07385a	0.08835a
2	0.2129b	0.1133b	0.08645b	0.08270b	0.06575a	0.08930a
3	0.1963c	0.1305a	0.08375b	0.08145b	0.06900a	0.1513a
5	---	0.1055c	0.1011a	0.07910b	0.08525a	0.07485a

Column values followed by the same letter are not siginificantly different (P≤0.10)
Standard deviations are shown in Appendix C3

Appendix C7: Simple Effect of Ca^{2+} on the tan Delta of the BSA Gel in 1% Phytic Acid

	tan Delta					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	0.3042a	0.1243b	0.09690b	0.09190b	0.07050a	0.05825b
0.005	0.2363a	0.1420a	0.1097a	0.1004b	0.1115a	0.08050a
0.01	0.2692a	0.1205b	0.08840c	0.1125a	0.07385a	0.08835a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix C1 to C3

Appendix C8: Simple Effect of Ca^{2+} on the tan Delta of the BSA Gel in 2% Phytic Acid

	tan Delta					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	0.2302a	0.1363a	0.1014a	0.07380c	0.06060a	0.06325a
0.005	0.2155b	0.1161b	0.09255b	0.09185a	0.08785a	0.08165a
0.01	0.2129b	0.1133b	0.08645c	0.08270b	0.06575a	0.08930a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix C1 to C3

Appendix C9: Simple Effect of Ca^{2+} on the tan Delta of the BSA Gel in 3% Phytic Acid

	tan Delta					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	---	0.1152b	0.07865b	0.06780c	0.06735b	0.04970a
0.005	0.2176a	0.1365a	0.1045a	0.1102a	0.1193a	0.08055a
0.01	0.1963b	0.1305a	0.08375b	0.08145b	0.06900b	0.1513a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix C1 to C3

Appendix C10: Simple Effect of Ca^{2+} on the tan Delta of the BSA Gel in 5% Phytic Acid

	tan Delta					
M Ca^{2+}	pH4	pH5	pH6	pH7	pH8	pH9
0	---	0.1241b	0.08715b	0.07455a	0.07545b	0.06760b
0.005	---	0.1321a	0.1013a	0.07815a	0.08185ab	0.08610a
0.01	---	0.1055c	0.1011a	0.07910a	0.08525a	0.07485ab

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix C1 to C3

Appendix D: The Binding Extent of Canola 12S Globulin with Phytic Acid (mole of phytic acid/mole of 12S)

Dependent Variable: Binding Extent

Source	DF	Sum of Squares	Mean Square	F Value	Pr. > F
Model	11	2211.48	201.044	27.16	0.0001
Error	12	88.8347	7.40289		
Corrected Total	23	2300.32			

R-Square	C.V.	Root MSE	Binding Extent Mean
0.961382	58.1533	2.72083	4.67871

Source	DF	Type III SS	Mean Square	F Value	Pr. > F
pH	2	1670.95	835.473	112.86	0.0001
Phytic acid	1	128.054	128.054	17.30	0.0013
pH*Phytic acid	2	67.8766	33.9383	4.50	0.0332
Ca^{2+}	1	146.227	146.227	19.75	0.0008
pH*Ca^{2+}	2	35.3101	17.6550	2.38	0.1343
Phytic acid* Ca^{2+}	1	51.6890	51.6890	6.98	0.0215
pH*Phytic acid*Ca^{2+}	2	111.3809	55.6904	7.52	0.0076

Appendix D1: Simple Effect of pH on Binding of Phytic Acid to Canola 12S Globulin

	Binding Extent			
	0M Ca^{2+}		0.01M Ca^{2+}	
pH	2% PA	5% PA	2% PA	5% PA
5	13.14±0.07425a	20.51±0.5926a	9.842±0.8934a	17.22±1.320a
7	2.471±1.58b	8.858±4.312b	-0.8925±1.192b	5.929±5.752b
9	-5.507±1.705c	3.404±4.541b	-4.846±1.103c	-13.99±2.407c

Column values followed by the same letter are not significantly different (P≤0.01)

Appendix D2: Simple Effect of Calcium on Binding of Phytic Acid to Canola 12S Globulin

	Binding Extent					
	2% PA			5% PA		
M Ca^{2+}	pH5	pH7	pH9	pH5	pH7	pH9
0	13.14a	2.471a	-5.507a	20.51a	8.858a	3.404a
0.01	9.842b	-0.8925a	-4.846a	17.22b	5.929a	-13.99b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix D1

Appendix D3: Simple Effect of Phytic Acid Concentration on Binding of Phytic Acid to Canola 12S Globulin

	Binding Extent					
	0M Ca^{2+}			0.01M Ca^{2+}		
% PA	pH5	pH7	pH9	pH5	pH7	pH9
2	13.14b	2.471a	-5.507a	9.842a	-0.8925a	-4.846a
5	20.51a	8.858a	3.404a	17.22b	5.929a	-13.99b

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix D1

Appendix E: The Storage Modulus (G') of Canola 12S Globulin Gel

Dependent Variable: Storage Modulus, G'

Source	DF	Sum of Squares	Mean Square	F Value	Pr. > F
Model	17	83777.5	4928.09	9.83	0.0001
Error	18	9020.56	501.142		
Corrected Total	35	92798.1			

R-Square	C.V.	Root MSE	Storage Modulus Mean
0.902794	36.5785	22.3862	61.2005

Source	DF	Type III SS	Mean Square	F Value	Pr. > F
pH	2	70174.3	35087.1	70.01	0.0001
Phytic acid	2	2961.89	1480.95	2.96	0.0777
pH*Phytic acid	4	4013.72	1003.43	2.00	0.1373
Ca^{2+}	1	809.700	809.700	1.62	0.2199
pH*Ca^{2+}	2	1088.64	544.321	1.09	0.3586
Phytic acid* Ca^{2+}	2	1954.49	977.244	1.95	0.1712
pH*Phytic acid*Ca^{2+}	4	2774.82	693.704	1.38	0.2789

Appendix E1: Simple Effect of Effect of pH on the G'of the Canola 12S Globulin Gel

	G'					
	0M Ca^{2+}			0.01M Ca^{2+}		
pH	0% PA	2% PA	5% PA	0% PA	2% PA	5% PA
5	18.08±9.564b	67.31±32.14ab	47.88±4.258b	22.34±16.46b	79.56±26.27b	49.85±25.87b
7	25.80±19.19b	10.27±5.180b	12.82±2.554b	14.07±4.739b	10.85±1.053c	17.95±1.630b
9	108.1±15.70a	95.09±37.32a	122.8±43.54a	114.9±19.95a	173.2±42.06a	110.8±10.88a

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix E2: Simple Effect of Phytic Acid Concentration on the G'of the Canola 12S Globulin Gel

	G'					
	0M Ca^{2+}			0.01M Ca^{2+}		
% PA	pH5	pH7	pH9	pH5	pH7	pH9
0	18.08b	25.80a	108.1a	22.34b	14.07ab	114.9a
2	67.31a	10.27a	95.09a	79.56a	10.85b	173.2a
5	47.88ab	12.82a	122.8a	49.85ab	17.95a	110.8a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix E1

Appendix E3: Simple Effect of Calcium on the Storage Modulus of the Canola 12S Globulin Gel

	G'								
	0% PA			2% PA			5% PA		
M Ca^{2+}	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	18.08a	25.80a	108.1a	67.31a	10.27a	95.09a	47.88a	12.83a	122.8a
0.01	22.34a	14.07a	114.9a	79.56a	10.85a	173.2a	49.85a	17.95a	110.8a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix E1

Appendix F: The tan Delta of Canola 12S Globulin Gel

Dependent Variable: tan Delta

Source	DF	Sum of Squares	Mean Square	F Value	Pr. > F
Model	17	0.0362911	0.00213477	3.36	0.0072
Error	18	0.0114316	0.00063509		
Corrected Total	35	0.0477227			

R-Square	C.V.	Root MSE	tan Delta Mean
0.760458	18.2022	0.0252010	0.138450

Source	DF	Type III SS	Mean Square	F Value	Pr. > F
pH	2	0.0247823	0.0123912	19.51	0.0001
Phytic acid	2	0.00043716	0.00021858	0.34	0.7134
pH*Phytic acid	4	0.000848000	0.00021200	0.33	0.8516
Ca^{2+}	1	0.00179493	0.00179493	2.83	0.1100
pH*Ca^{2+}	2	0.00375298	0.00187649	2.95	0.0777
Phytic acid* Ca^{2+}	2	0.00127598	0.00063799	1.00	0.3858
pH*Phytic acid*Ca^{2+}	4	0.00339978	0.00084994	1.34	0.2941

Appendix F1: Simple Effect of pH on the tan Delta of the Canola 12S Globulin Gel

	tan Delta					
	0M Ca^{2+}			0.01M Ca^{2+}		
pH	0% PA	2% PA	5%PA	0% PA	2% PA	5% PA
5	0.1630± 0.0223a	0.1792± 0.0095a	0.1530± 0.0093a	0.1827± 0.0167a	0.1757± 0.0103a	0.1635± 0.0037a
7	0.1361± 0.0002ab	0.1824± 0.0763a	0.1643± 0.0532a	0.1474± 0.0293a	0.1107± 0.0147b	0.1025± 0.0124b
9	0.1156± 0.0029b	0.1045± 0.0016a	0.1120± 0.0035a	0.0909± 0.0091b	0.1013± 0.0166b	0.1080± 0.0094b

Column values followed by the same letter are not significantly different (P≤0.10)

Appendix F2: Simple Effect of Phytic Acid Concentration on the tan Delta of the Canola 12S Globulin Gel

	tan Delta					
	0M Ca^{2+}			0.01M Ca^{2+}		
% PA	pH5	pH7	pH9	pH5	pH7	pH9
0	0.1630a	0.1361a	0.1156a	0.1827a	0.1474a	0.09085a
2	0.1792a	0.1824a	0.1045b	0.1757a	0.1107a	0.1013a
5	0.1530a	0.1643a	0.1119a	0.1635a	0.1025a	0.1080a

Column values followed by the same letter are not significantly different (P≤0.10)
Standard deviations are shown in Appendix F1

Appendix F3: Simple Effect of Calcium on the tan Delta of the Canola 12S Globulin Gel

	tan Delta								
	0% PA			2% PA			5% PA		
Molar Ca^{2+}	pH5	pH7	pH9	pH5	pH7	pH9	pH5	pH7	pH9
0	0.1630a	0.1361a	0.1156a	0.1792a	0.1824a	0.1045a	0.1530a	0.1643a	0.1119a
0.01	0.1827a	0.1474a	0.09085b	0.1757a	0.1107a	0.1013a	0.1635a	0.1025a	0.1080a

Column values followed by the same letter are not significantly different ($P \leq 0.10$)
Standard deviations are shown in Appendix F1