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

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Amy Wan-sau Wong

In Partial Fulfilment of the

Requirements for the Degree

of

Master of Science

Food Science Department

1995

ISBN 0-612-13568-3

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

BY

AMY WAN-SAU WONG

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

MASTER OF SCIENCE

The University of Manitoba requires the signatures of all persons using or photocopying this thesis. Please sign below, and give address and date.

ACKNOWLEDGEMENTS

I wish to take this opportunity to thank, all too briefly, those who helped me to finish my research project. First of all, I would like to express my great appreciation to my advisor, Dr. S. Arntfield for all her guidance and continuing support. Special thanks are extended to Drs N.A.M. Eskin and M.A.H. Ismond for being members of my thesis committee.

I am deeply grateful to Mrs. A. Bernatsky, Mr. J. Rogers and R. Roller for their technical assistance and advice in the laboratory and the computer room.

Finally, I wish to express my gratitude to my fellow colleagues, especially Shika, for their cheerful repartee.

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.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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

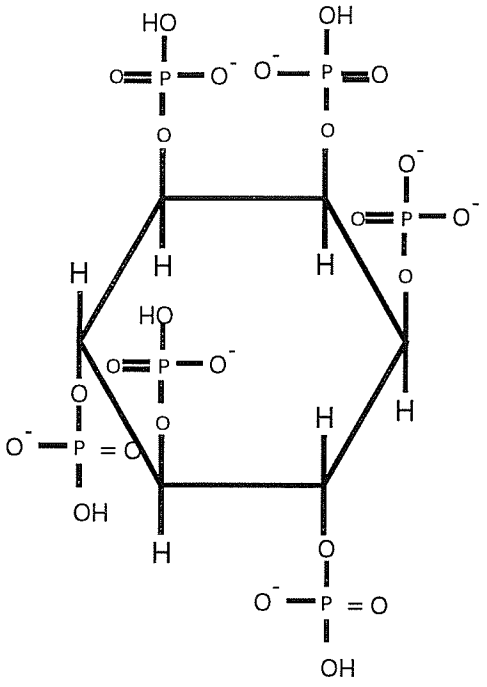
Phytic acid, a 6-carbon ring consisting of six phosphates, is one of the polyphosphorylated inositols commonly found in nature. It has been found in the greatest amounts in cereal, legumes and nuts. In general, phytate constitutes about 1 to 2% by weight of many cereals and oilseeds. The term phytin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

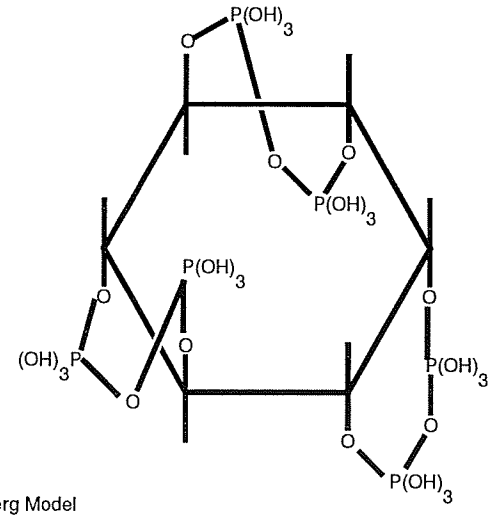
1. Structure and Chemistry

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.

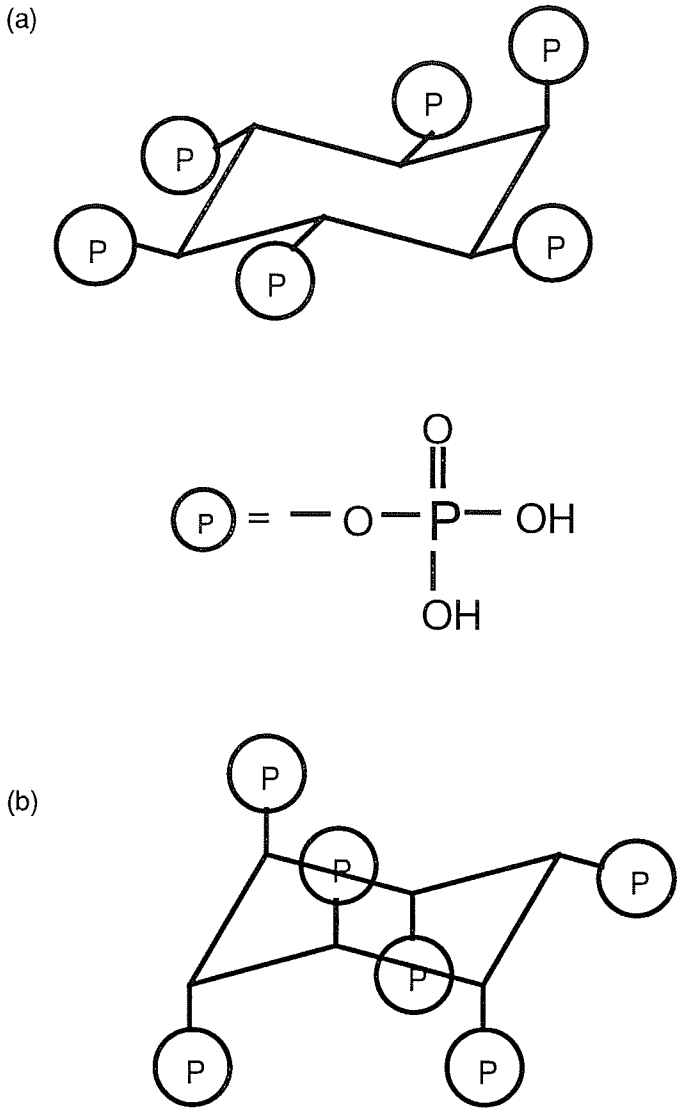


Figure 2. Two Possible Configurational Isomers of the Anderson Structure (Johnson and Tate, 1969; Blank et al., 1971)

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,

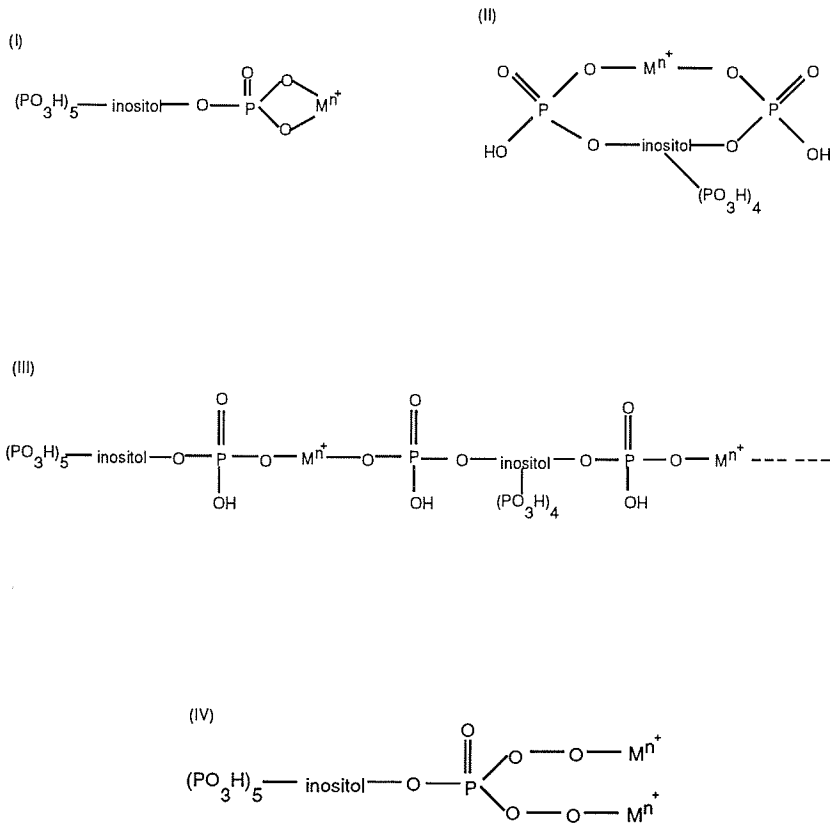


Figure 3. Four Theoretical Structures of Metal Phytate
(Nolan et al., 1987)

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