CHAPTER 2

LITERATURE REVIEW

2.1 Phytic acid

2.1.1 Physiological function of phytic acid

Phytic acid (*myo*-inositol 1, 2, 3, 4, 6-hexakisphosphate), is the major storage form of phosphorus in cereals, legumes and oilseeds as shown in Table 1. It serves several physiological functions in seed as phosphorus storage, a source of cations, energy storage, a source of *myo*-inositol (a cell wall precursor) and initiation of dormancy because it is expected as a natural antioxidant for protecting other storage substances like starch and lipids and serves several other unknown functions in seed (Reddy *et al*., 1989). The salts of phytic acid are described as phytates. In natural, it exists as a complex with cation (Ca^{2+}, Mg^{2+}, Zn^{2+} and K^{+}) or protein bodies. *Myo*-inositol hexakisphosphate is commonly called phytic acid for the free acid, phytate for the salt and phytin for the calcium and/or magnesium salt (Onumpai, 2005).

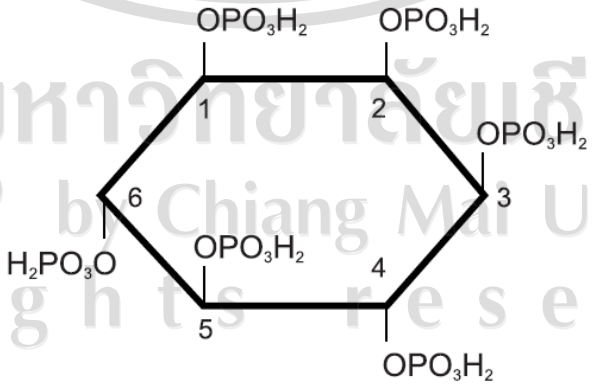

Figure 1 Structure of phytic acid (Baruah *et al*., 2004)

Table 1 Phytate contents in plants or plant products

	Total P (g/kg)	Phytate-P (g/kg)	Proportion (%)
Cereals			
Wheat grain	3.07	2.19	71.6
Oat	3.60	2.10	59.0
Corn grain	2.62	1.88	71.6
Barley grain	3.21	1.96	61.0
Sorghum grain	3.01	2.18	72.6
Rye	3.05	1.95	63.9
Oilseed meals			
Canola meal	9.72	6.45	66.4
Cottonseed meal	10.02	7.72	77.1
Corn glutton meal	4.24	2.67	63.0
Rapeseed meal	9.60	6.34	66.0
Soybean meal	6.49	3.88	59.9
By-products			
Rice bran	17.82	14.17	79.5
Wheat bran	10.96	8.36	76.3

Source: Cao *et al*. (2007)

2.1.2 Chemical structure of phytic acid

The conformational structures for phytic acid have been derived from X-ray analysis (Blank *et al*., 1971) and ^{31}P-NMR (Johnson and Tate, 1969). Johnson and Tate suggested that the phosphate at 2-position is in axial position, the other phosphates being in an equatorial position. In contrast, Blank *et al*. (1971) concluded that the phosphate groups at the 1-, 3-, 4-, 5-, and 6-positions are axial, that at the 2-position being equatorial. Data of Costello *et al*. (1976) supports the conformation suggested by Johnson and Tate (1969). This energetically most favorable conformation of phytic acid is shown in Figure 1. Costello *et al*. (1976) also determined pK_a values for dissociating groups of phytic acid using ^{31}P-NMR and pH titration methods. They concluded that six groups were in the strong acid range (pK_a

1.1 to 2.1), one in the weak acid range (pK_a 5.70), two with pK_a 6.80 to 7.60, and three in the very weak acid range (pK_a 10.0 to 12.0). This suggests that phytic acid has a strong potential for complexing multivalent cations and positively charged proteins, since it exists as a strongly negatively charged molecule over a wide pH range.

2.1.3 Occurrence of phytic acid

Phytic acid occurs primarily as salts of mono- and divalent cations (e.g. potassium, magnesium salt in rice and calcium-magnesium-potassium salt in soybeans) in discrete regions of cereal grains and legumes. It accumulates in seeds and grains during ripening, accompanied by other storage substances such as starch and lipids. In cereals and legumes phytic acid accumulates in the aleurone particles and globoid crystals, respectively (Reddy *et al*., 1989). The endosperm of wheat and rice kernels is almost devoid of phytate, as it concentrates in the germ and aleurone layers of the cells of the kernel. Ferguson and Bollard (1976) found that 99% of the phytate in dry peas was in the cotyledons and 1% in the embryo taxis. The highest amount of phytate among cereals is found in maize (0.83 - 2.22%) and among legumes in dolique beans (5.92 - 9.15%) (Reddy *et al*., 1989).

2.1.4 Antinutritive effect of phytic acid

Phytic acid has been shown to have a strong antinutritive effect (Pallauf and Rimbach, 1996). This effect is based on the unusual molecular structure of phytic acid. At complete dissociation, the six phosphate groups of phytic acid carry a total of twelve negative charges.

2.1.4.1 Effect on mineral utilization

Phytic acid effectively binds different mono-, di-, and trivalent cations and their mixtures to form insoluble complexes (Reddy *et al*., 1989). The formation of insoluble phytate-mineral complexes in the intestinal tract prevents mineral absorption. This reduces the bioavailability of essential minerals (Davies, 1982). Zinc appears to be the trace element of which the bioavailability is most influenced by phytic acid. Rimbach and Pallauf (1992) showed that graduated phytic acid

supplementations had a negative influence on apparent Zn^{2+} absorption and life weight gain of growing rats.

2.1.4.2 Effect on protein digestibility

Phytic acid interacts with proteins over a wide pH range to form phytate-protein complexes. At a low acidic pH, phytic acid has a strong negative charge due to total dissociation of phosphate groups. Under these conditions a negative influence of phytic acid on the solubility of proteins can be expected because of the ionic binding between the basic phosphate groups of phytic acid and protonized amino acid (lysyl, histidyl and arginyl) residues (De Rham and Jost, 1979; Fretzdorff *et al*., 1995). Under acidic conditions phytic acid is likely to bind tightly to plant proteins, since the isoelectric point of plant proteins is generally around pH 4.0 - 5.0. In the intermediate pH range (6.0 to 8.0) both phytic acid and plant proteins have a net negative charge. However, under these conditions complex formation occurs between phytic acid and proteins. Possible mechanisms include direct binding of phytic acid to protonate ε-NH_2 terminal groups and ε -NH_2 groups of lysine residues, and a multivalent cation-mediated interaction (Cheryan, 1980). By binding to plant proteins, phytic acid decreases their solubility and digestibility, therefore also reduces their nutritive value. According to minerals and protein complexes, phytic acid interacts with enzymes such as trypsin, pepsin, α-amylase and β-galactosidase, resulting in a decrease in the activity of these important digestive enzymes (Deshpande and Cheryan, 1984; Singh and Krikorian, 1982; Inagawa *et al*., 1987).

2.1.4.3 Environmental effect

Since, monogastric animals are not able to metabolize phytate, excess inorganic phosphate is added to their feed to meet efficacy of phosphorus and also ensure the animal growth. Therefore, high amount of phosphate and undigested phytic acid are excreted in manure and liquid effluent which are distributed to environment. If they are run off to lakes and rivers, it is most available for phytoplankton growth and can be the main cause of eutrophication

2.2 Phytase

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the hydrolysis of *myo*-inositol hexakisphosphate (phytic acid) to inorganic monophosphate and lower *myo*-inositol phosphates and in some cases to free *myo*-inositol as shown in Figure 2. It was first discovered in 1907 (Wodzinski and Ullah, 1996). Vohra and Satyanarayana (2003) reported that phytase activity was first detected in rice bran nearly a century ago and was developed to be a feed enzyme since 1962 in North America. The Enzyme Nomenclature Committee of International Union of Biochemistry distinguishes two types of phytase: 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). This classification is based on the first phosphate group attacked by the enzyme. 3-phytase is typical for microorganisms and 6-phytase for plants (Kerovuo, 2000). Phytate-degrading enzyme also can be divided in two type based on their optimal pH. These are the acid phytase with a pH optimum around 5.0, and the alkaline phytase with a pH optimum around 8.0. Most of them belong to acid type (Knietzny *et al*., 2002). However, it has to be taken into account that microbial phytases of different source can differ in their bio-efficacy per unit. Phytase is widespread in nature and its activity has been reported in plant and animal tissue and in a variety of microorganisms (Kerovuo, 2000). Some of the reported phytases from various sources are summarized in Table 2.

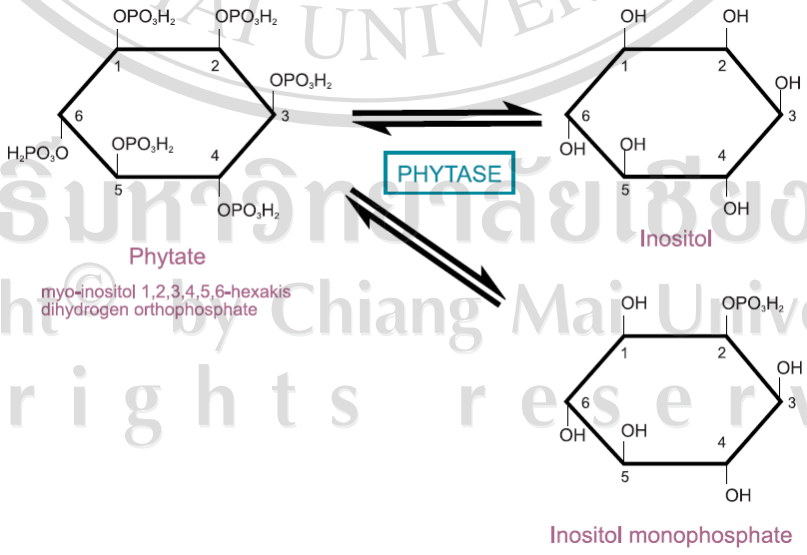

Figure 2 Action of phytase (Baruah *et al*., 2004)

Table 2 Phytases from various sources

Phytase source	Localization	References
Fungi		
A. niger NRRL 3135	EX	Shieh *et al.* (1969)
A. flavus	EX	Shieh and Ware (1968)
A. terrus	EX	Yamada *et al*. (1968)
A. carneus	EX	Ghareib (1990)
A. oryzae	EX	Shimizu (1993)
A. fumigatus	EX	Pasamontes *et al*. (1997)
Mucor sp.	EX	Shieh and Ware (1968)
Penicillum spp.	EX	Shieh and Ware (1968)
Penicillium caseoicolum	EX	Amano Pharmaceuticals (1995)
Rhizopus oligosporus	IN and EX	Sutardi and Buckle (1988)
Yeast		
Saccharomyces cerevisiae	EX	Nayini and Markakis (1984)
Schwanniomyces castelii	EX	Lambrechts *et al*. (1992)
Kluyveromyces fragilis	EX	Lambrechts *et al*. (1992)
Candida tropicalis	EX	Lambrechts *et al*. (1992)
Torulopsis candida	EX	Lambrechts *et al*. (1992)
Debaryomyces castelii	EX	Lambrechts *et al*. (1992)
Bacteria		
Bacillus subtilis	EX	Powar and Jagannathan (1982)
B. subtilis (natto)	EX	Shimizu (1992)
B. amyloliquefaciens	EX	Kim *et al*. (1998a,b)
Echerichia coli	IN[a]	Greiner *et al.* (1993)
Klebsiella aerogenes	IN	Tambe *et al*. (1994)
K. terrigena	IN	Greiner *et al*. (1997)
K. oxytoca	IN	Jareonkitmongkol *et al.* (1997)
Pseudomonas sp.	EX	Irving and Cosgrove (1971)
Enterobacter sp.	EX	Yoon *et al*. (1996)
Citrobacter freundii	IN	Delucca *et al*. (1992)

Table 2 Phytases from various sources (Continued)

Phytase source	Localization	References
Plants		
Maize, germinated	IN	Laboure *et al.* (1993)
Soybean seeds	IN	Gibson and Ullah (1988)
Legume seeds	IN	Scott (1991)
Typha latifolia, pollen	IN	Hara *et al.* (1985)
Animals		
Rat, intestinal mouse	IN	Yang *et al.* (1991a,b)
Rat, liver	IN[b]	Craxton *et al.* (1997)
Paramecium	IN[b]	Freund *et al.* (1992)

Source: Kerovuo (2000)

EX: extra cellular

IN: intracellular

[a] periplasmic space

[b] endoplasmic reticulum

2.2.1 Source of phytase

Phytases can be derived from a number of sources including plants, animals and microorganisms as mentioned before. Recent research has shown that microbial sources are more promising for the production of phytases on a commercial level. Although several strains of bacteria, yeasts and fungi have been used for production under different conditions, two strains of *Aspergillus* sp., *A. niger* and *A. ficuum*, have most commonly been employed for its commercial production (Pandey *et al*., 2001). Shieh and Ware (1968) made one of the earliest approaches on microbial sources of phytases in 1968.

2.2.1.1 Microbial sources

2.2.1.1.1 Bacterial sources

Pandey *et al*. (2001) reported that several bacterial strains (wild or genetically modified) such as *Lactobacillus amylovorus, E. coli, B. subtilis, B. amyloliquefaciens, Klebsiella* sp., etc., have been employed for phytase synthesis.

Sreeramulu *et al*. (1996) evaluated 19 strains of lactic acid-producing bacteria of the genera *Lactobacillus* and *Streptococcus* for the production of extra-cellular phytase. A number of them exhibited the enzyme activity in the fermentation medium but *Lactobacillus amylovorus* B4552 produced the maximum amounts of phytase, ranging from 125-146 units/mL in SmF using glucose and inorganic phosphate. The findings were claimed significantly as *L. amylovorus* has potential in improving nutritional qualities of cereal and pulse-based food fermentations. A bacterial strain that produced extra-cellular phytase was isolated from soil near the roots of the leguminous plants and identified to be *Enterobacter* sp.. The optimum condition for phytase production in PSM medium was pH of 5.5 and 3 days of cultivation at 37°C (Yoon *et al*., 1996). Sunitha *et al*. (1999) optimized the medium for recombinant phytase production by *E. coli* BL21 using response surface methodology. A 23 central composite experimental design was used to study the combined effects of the medium components, tryptone, yeast extract and NaCl. The optimized medium with glucose showed the highest phytase activity of 2250 units/l. A genetically modified *B. subtilis* produced extra-cellular phytase (2.0 units/ml), which constituted over 90% of the total protein. The yield was 100-fold higher than the wild type *B. amyloliquefaciens* DS11 (Kim *et al*., 1999a). A bacterial strain, *Bacillus* sp. KHU-10, produced a high level of an extra-cellular phytase in a maltose, peptone and beef extract medium. Under the optimized conditions, the highest phytase production (0.2 unit/ml), was obtained after 4 days of fermentation (Choi *et al*., 1999).

2.2.1.1.2 Yeast sources

Phytase production using yeast cultures has generally been carried out in SmF systems. The strains used include *Schwanniomyces castellii, S. occidentalis, Hansenula polymorph, Arxula adeninivorans, Rhodotorula gracilis*, etc. In a continuous culture using a strain of *S. castellii*, phytase production increased with pH and dilution rate. It decreased when the phytic acid or phosphate content increased. Mayer *et al*. (1999) developed an efficient process for the low-cost production of phytases using *Hansenula polymorpha*. Glucose or glucose syrups were used as main carbon sources during fermentation. Compared with the process using glycerol the use of glucose led to a reduction of more than 80% in the raw materials costs. In addition, exceptionally high concentrations of active enzyme (up to 13.5 g/l) were obtained in

the medium, with phytase representing over 97% of the total accumulated protein. These levels greatly exceed those reported so far for any yeast-based expression system (Pandey *et al*., 2001).

2.2.1.1.3 Fungal sources

Ahmad *et al*. (2000) used maize starch-based medium for the production of phytase in SmF using *A. niger*. Activity of the enzyme was found to be 1.075 phytase units per min per ml of the crude culture filtrate at pH 5.5 and 40°C (10 days). Extra-cellular phytase produced by *Aspergillus* sp. 5990 showed a five fold higher activity in liquid culture when compared with cultures of *A. ficuum* NRRL 3135. SmF was carried out at 35°C, pH 7 for 4 days. The phytase had a higher optimum temperature for its activity than the commercial enzyme, Natuphos, from *A. ficuum* NRRL 3135 (Kim *et al*., 1999c). SSF was employed for phytase production using strains of *Aspergillus* sp.. Ebune *et al*. (1995) used canola meal for phytase production by *A. ficuum*. Optimum substrate moisture was 64%. Age of the inoculum had a profound effect on enzyme synthesis by the culture. Using a strain of *A. carbonarius* on canola meal, Alasheh and Duvnjak (1995) found 53-60% moisture as the optimum. Biomass growth and enzyme synthesis were increased when glucose at lower concentrations (6 g) and surfactants such as Na-oleate or Tween-80 were added in the medium (Alasheh and Duvnjak, 1994a, b).

2.2.1.2 Plant sources

Phytase occurs widely in the plant kingdom. Phytase has been isolated and characterized from cereals such as triticale, wheat, maize, barley and rice and from beans such as navy beans, mung beans, dwarf beans and California small white beans. Phytase activity has also been detected in white mustard, potato, radish, lettuce, spinach, grass and lily pollen (Dvorakova, 1998). Laboure *et al*. (1993) purified and characterized phytase from germinating maize seedlings (Zea mays), and the cDNA coding for this phytase was cloned (Maugenest *et al*., 1997). This cDNA was used to screen a maize genomic library and two distinct genes were isolated and sequenced.

2.2.1.3 Animal sources

Phytase has been found to exist in monogastric animals (Bitar and Reinhold, 1972; Copper and Gowing, 1983; Yang *et al*., 1991a; Chi *et al*., 1999). Generally, however, intestinal phytase does not play a significant role in food-derived phytate digestion in monogastrics (Williams and Taylor, 1985). Craxton *et al*. (1997) cloned and expressed a rat hepatic multiple inositol polyphosphate phosphatase (MIPP) having phytase activity. The MIPP mRNA was present in all rat tissues examined, but was most highly expressed in kidney and liver. A phytase-like enzyme was also described in the protozoan *Paramecium* (Freund *et al*., 1992).

2.2.2 Commercial phytases

Several distinct microbial phytase products are now commercially available. Phytase feed enzymes may be included in fish feed as powder, granulate or liquids, via post-pelleting or pre-treatment to avoid thermostability problems at high pelleting temperatures (>80°C). Phytases produced on commercial scale are either derived from fungal strains mutated or by using recombinant DNA technology. The three commonly used phytase feed enzymes are derived from *A. niger* which is a 3-phytase, *Peniophora lycii* and *E. coli*, which are 6-phytases. The fungal phytase has the higher thermo-stability and lower optimum pH range than the bacterial phytase (Liebert *et al*., 2005). In general, different sources of phytases have different characteristics, should be considered before applied in feed.

Table 3 summarizes published phytase properties and commercial information from different authorized phytase companies. Phytase activities are determined on the basis of inorganic-P released from phytate. Due to obvious differences with respect to cultivation conditions and slight differences with respect to phytase assay conditions, a comprehensive comparison and evaluation of the production strains is difficult. According to the market research report (Hou, 2001), phytases from Europe and North America are more competitive than phytases produced by Asian companies. The reason is that the former has higher activity per unit. Usually, the activity of powder phytase from Europe and North America can reach 40,000–4,000,000 units/g. Their lipid phytase activity is over than 40,000,000 units/ml. In addition, these phytases have wider pH range and temperature tolerance

than those from Asian companies (Zhan *et al*., 2004). For example, Natuphos enzyme produced by BASF Company can maintain 75% activity under 75°C for 15 min (Wang *et al*., 2001). Allzyme phytase produced by Alltech Company can keep more than 60% of activity when pH reaches above 6.5 or below 2.5. The market prices of phytases from the former companies (approximately $12.5–15 per kilogram) are also more stable than those from the later (Zhan *et al*., 2004). In contrast, the phytases from many Asian companies are newly developed, such as in China, Japan and South Korea. Their formulation methods are still immature. The phytase activity is usually around 500–5000 units/g or 5000–50,000 units/ml. And the prices of these phytase fluctuate around $3–10 per kilogram (Wang *et al*., 2001).

Table 3 Commercial production information of microbial phytases

Companies	Countries	Phytase sources	Production strains	Trademarks
AB Enzyme	Germany	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytaae
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lyciii*	*P. lyciii*	Bop-Feed Phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*P. simplicismum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*A. awamori*	*T. reesei*	Finase
Novozyme	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme® Roxazyme®

Source: Stafan *et al*. (2005) and Hou *et al*. (2001)

2.3 Regulation of phytase formation

In bacteria, phytase is an inducible enzyme and its expression is subjected to a complex regulation, but phytase formation is not controlled uniformly among different bacteria. Until now, phytase production was studied in some detail only in *Escherichia coli* and *Raoultella terrigena*. In non-limiting media the formation of both the *Escherichia coli* and the *Raoultella terrigena* phytase was turned off in exponentially growing bacteria and started as soon as the cultures entered the stationary phase. Because the synthesis of the enzymes started as soon as the growth rate began to fall, it was suggested that either a nutrient or an energy limitation, known to occur in the stationary phase, could be at the origin of its induction. Among the nutrient limitations tested, only carbon starvation was able to provoke an immediate synthesis of the *Raoultella terrigena* phytase, whereas in *Escherichia coli*, phytase synthesis was triggered, when bacteria were starved for inorganic phosphate, while carbon, nitrogen and sulfur limitation were ineffective. A tight regulatory inhibition of phytase formation by inorganic phosphate levels was generally observed in all microbial phytase producers, including moulds, yeast and bacteria, with the exception of *Raoultella terrigena* and the rumen bacteria. The repression of phytase synthesis by inorganic phosphate seems to be less significant with higher medium composition complexities. It is not known, however, what components in the complex media account for the reduced repression.

In *Escherichia coli*, the primary response to the limitation of a specific nutrient was shown to be an activation of a certain set of genes that allow a better uptake of the nutrient present in low concentration or the utilization of other substances that belong to the same class of nutrients. These nutrient-specific systems include the cyclic AMP (cAMP) and its receptor the catabolite activator protein (CAP) for the use of alternative carbon sources, the NtrB/NtrC/σ^{54} regulon that is induced under nitrogen limitation, and the PhoB/PhoR regulon that is induced under phosphorus limitation. However, if the environment is totally exhausted for an essential nutrient, the cells enter into the stationary phase. The formation of several dozen proteins is stimulated during transition into stationary phase and a core set of proteins is induced regardless of the class of nutrient for which the cells are starved. As mentioned above, phytase formation in *Escherichia coli* is induced in non-limiting

media upon entry into the stationary phase and under anaerobic conditions. The expression of the phytase-encoding gene *appA* was shown to be strongly dependent on the *rpoS*-encoded sigma factor σ^S, which has been identified as a central regulator for many stationary-phase-responsive genes. Very often σ^S-dependent genes are regulated by several promoters and only one of them is controlled by σ^S. Thus, not all genes identified as σ^S-controlled are entirely dependent on σ^S for expression. In minimal medium, starvation for phosphate, but not for glucose or ammonia, resulted in a strong stimulation of *rpoS* expression followed by an increase in phytase activity. In addition, phytase expression depends on the nature of the carbon source used for growth. Glucose, which is known to cause catabolite repression, has been widely used to improve phytase production. That cAMP-CAP, rather than the carbon source itself, are directly involved in this regulation was shown in *Escherichia coli*. Synthesis of the phytases in both *Escherichia coli* and *Raoultella terrigena* have been reported to be negatively regulated by cAMP, which is suggested to be involved in the amphibolic metabolism of glucose and galactose as well as directly or indirectly in controlling the expression of an important stationary growth regulator. For example, *rpoS* transcription in *Escherichia coli* was described to be negatively controlled by the cAMP-CAP complex. For several *Raoultella* sp. it was reported that phytate is needed to induce phytase production. Substrate induction was also found in *Mitsuokella jalaludinii*, whereas phytate had no effect on the formation of phytase in *Escherichia coli*. Phytase formation in *Pseudomonas* sp. and *Raoultella aerogenes* was reported to be significantly induced in the presence of *myo*-inositol as the sole carbon source. In the other *Raoultella* sp. studied *myo*-inositol was ineffective (Konietzny and Greiner, 2004).

2.4 *In vivo* function of bacterial phytase

The complex mode of regulation does not shed much light on the role of bacterial phytases. The stationary phase induction suggests that phytase is not required for balanced growth, and that this enzyme may be synthesized in response to a nutrient or energy limitation. This suggestion could also explain why there is, with the exception of sourdough bacteria, no clear evidence for lactic acid bacteria with phytase-producing ability. Lactic acid bacteria are adapted to environments rich in

nutrients and energy and therefore, it never may have been an evolutionary selection for lactic acid bacteria with the capability to produce a phytase. The efficient induction or depression of phytase formation by phosphate starvation in most bacteria raises the question of a possible role in providing the cell with phosphatehydrolysed from molecules such as phytate. This hypothesis is, for example, supported by the identification of a phytase in the stalk of *Caulobacter crescentus*, a gram-negative alphapurple proteobacterium, which is an oligotroph that lives in aquatic environments dilute in nutrients. Phosphate is the limiting nutrient in the environments in which *Caulobacter* is found and one of the hypothesized functions of the stalk is phosphate uptake. Stalks elongate when phosphate is limiting and increasing the surface area available for phosphate uptake as well as the presence of a phytase would allow the uptake of the organically phosphate by the stalk. In addition, ruminants seem to digest phytate through the action of phytase produced by microbial flora in the rumen. In contrast to most other bacteria, anaerobic rumen bacteria are capable of tolerating a high level of phosphate without any negative impact on phytase production. This unique ability leads to a more efficient phytate hydrolysis in the rumen, even under the high phosphate levels in the rumen fluid of ruminants fed concentrated feed. The phosphate generated by splitting of phytate is utilized by both the microbial flora and ruminant host. Because phytase formation was observed when bacterial cells had to adapt to environmental fluctuations imposed before the onset of growth or when actively growing cells are stressed, it was suggests that phytase could be involved in a signal transduction mechanism of metabolic regulation. Several *myo*-inositol phosphates have recently been identified in gram negative bacteria and are probably involved in signal transduction. For instance, *Salmonella dublin* excretes an *myo*inositol polyphosphate 4-phosphatase, which contributes to its virulence by subverting cellular *myo*-inositol phosphate signaling reactions. Furthermore, two proteins with *myo*-inositol monophosphate phosphatase activity implicated in the control of gene expression have also been reported in *Escherichia coli* and *Rhizobium leguminosarum*. In higher plants phytases occur predominantly in grains, seeds and pollen. These enzymes were reported to be responsible for phytate degradation during germination to make phosphate, minerals and *myo*-inositol available for plant growth and development. A low phytase activity is also found in the plant root. Root phytase

has been suggested as one of the mechanisms of plants to improve utilisation of soil phosphate. Organic forms of phosphate generally account for at least 50% of total soil phosphate and it is known that a major component occurs in form of inositol penta- and hexakisphosphates. Due to the low phytase activity in roots and the inability of phytase secretion into the rhizosphere, however, phytate appears to be only poorly utilised by plants. Thus, it was suggested, that soil microorganisms colonizing the plant rhizosphere and producing extracellular phytase activity, such as *Bacillus* and *Enterobacter* ssp., could act as plant growth promoting rhizobacteria (PGPR) by making phytate phosphate available to the plant. Recently, the importance of phosphate availability from soil phytate for plant nutrition under phosphate limitation was demonstrated by enabling the plants to utilise phytate phosphate through expression of extracellular secreted *Aspergillus* phytase in the plant root and by adding of purified phytase as well as soil microorganisms expressing extracellular phytase activity to the rooting medium (Konietzny and Greiner, 2004).

2.5 Application of phytase

2.5.1 Feed Application

Ruminants digest phytase through the action of phytase produced by microbial flora in the rumen. The anaerobic gut fungi and bacterial present in the microflora of ruminants are responsible for the primary colonization of plant material within the rumen. The inorganic phosphate hydrolyzed from phytate by phytases is utilized by both the microflora and the ruminant host. The situation is different with monogastric animals. The monogastrics, such as pig, poultry and fish are unable to metabolize phytic acid, since they lack gastrointestinal phytase. Therefore, inorganic phosphate is added to their feed to meet the phosphate requirement. This increased costs and contributes to phosphate pollution problems. The supplement of animal feed with phytase enables the assimilation of phosphate in the feed ingredients and diminishes the amount of phosphate in the manure and subsequently reaching the environment. The effect of feeding phytase to animals on pollution has been quantitatively determined. The use of phytase as feed additive has been approved in 22 countries. The FDA (The Food and Drug Administration) has approved the

phytase preparation as GRAS (Generally Regarded As Safe) (Wodzinski and Ullah, 1996)**.** The use of phytase as a feed enzyme sets certain demands on the properties of the enzyme. Particularly, the enzyme should withstand high temperatures. This is because poultry and pig feed is commonly produced in pellet form, which ensure that the animals have a balanced diet and facilitates the preservation of enzyme-containing product in the feed industry (Kim *et al*., 1999a). During the pelleting process the temperatures may temporarily reach 90°C (Wyss *et al*., 1998).

2.5.2 Food application

A diet rich in cereal fibers, legumes and soy protein result in an increased intake of phytate. Vegetariens, eldery people consuming unbalanced food with high amounts of cereals, people in undeveloped countries who eat unleavened bread and babies eation soy-based infant formulas take in large amounts of phytate (Simell *et al*., 1989). Undigested phytate in the small intestine negatively affects the absorption of zinc, calcium, magnesium, and iron. It also reduces the digestability of dietary protein and inhibits digestive enzyme. Using Finase® phytase, Simell *et al*, (1989) reported the preparation of a phytate-free soy protein isolate with increased solubility at low pH (pH3) compared to the control soy protein isolate. Anno *et al*., (1985) eliminated phytate from soybean milk using wheat phytase. Additon of *A. niger* phytase to flour containing wheat bran increase iron absorption in humans (Sandberh *et al*., 1996). However, more studies should be investigated before accepting phytase as a food additive.

2.5.3 Preparation of *Myo*-Inositol phosphates

The increasing interest in inositol phosphate and phospholipids, which play a pivotal role in transmembrane signaling and mobilization of calcium from intracellular reserves, has resulted in a need for various inositol phosphate preparations (Billington, 1993). Furthermore, specific inositol triphosphates have been suggested to prevent or alleviate diseases or conditions associated with abnormal levels of Neuropetide Y (NPY) (Siren *et al*., 1992). Among others, these include inflammatory diseases such as arthritis and respiratory diseases such as asthma. The use of specific inositol triphosphates as pain killers has also been proposed (Siren,

1995). Surprisingly, the esters of inositol triphosphate have been shown to exert significant inhibitory effects against retroviral infections including HIV (Siren, 1998).

Pharmaceutical applications of specific *myo*-inositol phosphates have further increased interest in the preparation of these compounds. The chemical syntheses of *myo*-inositol phosphates include difficult protection and deprotection steps, and are performed at extreme temperatures and pressures (Billington, 1993). Since phytases hydrolyze myo-inositol hexaphosphate sequentially, the production of *myo*-inositol phosphate derivates and free *myo*-inositol using phytase is a potential alternative to chemical synthesis. The preparation of D-*myo*-inositol 1,2,6-trisphosphate, D-*myo*-inositol 1,2,5-trisphosphate, L-*myo*-inositol 1,3,4-trisphosphate and myo-inositol 1,2,3-trisphosphate, L-*myo*-inositol 1,3,4-trisphosphate and *myo*-inositol 1,2,3-trisphosphate by enzymeatic hydrolysisi of phytic acid by *S. cerevisiae* phytase has been described (Siren, 1986). Immobilized phytase have been used to produce various *myo*-inositol phosphates (Ullah and Philippy, 1998: Greiner and Konietzny, 1996).

2.5.4 Pulp and Paper Industry

It has been speculated that removal of plant phytic acid might be important in the pulp and paper industry. A thermostable phytase could have potential as a novel biological agent to degrade phytic acid during pulp and paper processing. The enzymatic degradation of phytic acid would no produce carcinogenic and highly toxic by-product. Therefore, the exploitation of phytases in the pulp and paper process could be environmentally friendly and would assist in the development of cleaner technologies (Liu *et al*., 1998).

2.6 Statistical experimental design

2.6.1 Plackett and Burman design

In an investigation, many factors are suspected to have effect on the process or do not and it takes a long time for testing each variable separately. The Plackett and Burman design is often used for screening important dependent variable at one time. According to this experiment can calculate the main of each factor on

process value by linear regression analysis. This design can not show the interaction between factors in the experiment. However, it is useful for preliminary study before process optimization. The Plackett and Burman design is a fractional factorial which each factor is set at two points as minimum (-) and maximum (-) level. The initial blocks which proper for the number of factor in experiment is used for set up first treatment as shown in Table 4. Then other treatments, which contain different level of each factor, are generated by cyclic construction (Onumpai, 2005).

2.6.2 Central composite design

The disadvantages of single variable optimization are time consuming; interaction between factors is perhaps overlooked and incapable to reach the true optimum. The regression analysis is a strategy for evaluate the relation between multi-factor and dependent or response value. In process optimization, the mathematical model from regression analysis is often used to predict the optimum value of process parameters. Central composite design (CCD) is one type to multiple linear regressions that commonly used with response surface methodology. The statistical program SXW version 7.0 is used for regression analysis. The obtained data are fitted to a second order polynomial model of CCD, which contained main effect and interaction term. The STATISTICA version 5.0 (Demonstration 2001, USA) is used for generate response surface graph. The optimal combinations of independent variables were calculated by optimizing the second order polynomial model using Mathcad 7.03 (Mathsoft Engineering & Education Inc.)(Onumpai, 2005).

Table 4 The initial block for Plackett and Burman design

N	Initial block
8	+ + + - + --
12	+ + - + + + - - - + -
16	+ + - - + + + + - + - + - - - - + + -
20	+ + - - + + + + - + - + - - - - + + -
24	+ + + + + - + - + + - - + + - - + - + - - - -

Table 5 Central composite design for two factors

Pentagon Design			Hexagon design			Octagon design		
Design point	X_1	X_2	Design point	X_1	X_2	Design point	X_1	X_2
1	1.000	0	1	1.000	0	1	-1	-1
2	0.309	0.951	2	0.500	0.866	2	1	-1
3	-0.809	0.588	3	-0.500	0.866	3	-1	1
4	-0.809	-0.588	4	-1.000	0	4	1	1
5	0.309	-0.951	5	-0.500	-0.866	5	1.4142	0
6	0	0	6	0	-0.866	6	-1.4142	0
			7	0	0	7	0	1.4142
						8	0	-1.4142
						9	0	0

2.7 Enzyme purification

2.7.1 Precipitation technique

Englard and Seifter (1999) reported the method for the precipitation of proteins for preparative purpose. Proteins can be precipitated by causing perturbation in the solvent with respect to pH, ionic strength and temperature. The properties of the solvent can also be modified by addition of high concentrations of certain salts or of miscible organic solvents. Addition of specific metal cations such as $Zn^{2+,}$ Cd^{2+} and Ba^{2+} or of compounds with bulky anions such as picrate, tannate, tungstate, molybdate, trichloroacetate, perchlorate and sulfosalicilate, can also precipitate proteins at appropriate pH values. Many of the last group of agents, because they also may be denaturing, are used principally for removal of proteins from a solution to allow analysis of nonprotein constituents. In thesis chapter emphasis is given to perturbants whose use has survived the introduction of more modern methods of protein purification such as column and affinity chromatography. Thus, the use of ammonium sulfate and of ethanol and/or acetone is considered in some detail.

2.7.1.1 History

Although the precipitation of proteins from biological fluids had been observed for hundreds of years (e.g., the precipitation of casein from milk by diluted acid), the fractionation and classification of proteins on the basis of solubilities began to receive serious attention only in the middle of the last century. From those studies

came a classification of proteins the nomenclature of which persists to this day with modifications in meaning. Thus, globulins were defined as proteins insoluble in water of dilute salt solutions and albumins as soluble in such media although precipitable by very high concentrations of ammonium sulfate. In a modern sense most of the globulins are in fact globular proteins as determined by physicochemical methods that determine size and shape of molecules but many of the water-soluble proteins such as hemoglobin and albumin are also globular.

Beyond classifying proteins on the basis of solubilities, early biochemists felt compelled to provide a theory for this remarkable precipitation behavior. On the hand, a great deal of attention was paid to the nature of the salts themselves: this is exemplified in the studies of Hofmeister, whose lyotropic series of electrolytes established an order of differential effects of various ions on solubility of proteins and other properties of biological systems. However, much of the proposed theory of precipitation also came from the then current knowledge of proteins. The theory was developed in an era in which proteins were considered to be colloids of undefined molecular nature with surface electrical charges that could be manipulated either to favor dispersion of to cause precipitation. Much of our current language and some of the concepts of this kind of protein chemistry reflect that historical development: salting in, salting out, isoelectric precipitation, zone of hydration, solvation and hydrophilic and hydrophobic characters. Proteins were not considered to be in true solution but rather in colloid dispersion stabilized by such forces as charge repulsion, interaction with solvent or exclusion of solvent. Destabilization and precipitation consisted of interfering with of neutralizing the stabilizing forces.

Eventually, proteins were shown to be molecules of definite molecular weight, size, shape, electrical properties and amino acid composition, capable of existing in true solution as compared to colloid dispersion. Concurrently, as metabolic pathways were being unraveled and defined, hundreds of new enzymes from a variety of biological sources had to be isolated and purified so that their molecular and catalytic properties could be determined. The art and science of protein fractionation by differential solubility and precipitability reached a pinnacle and a wide variety of sorbents such as alumina and calcium phosphate gels were used, most of the methods employed several steps of ammonium sulfate precipitation. The development of

column chromatography began in earnest in the late 1950s and soon dominated the field. From that time on, precipitation methods were supplanted in importance as a means of obtaining proteins in a final state of purity but found their present place as an adjunction for concentration of proteins and form one or two specific steps on the way to using chromatography. In this more limited capacity, precipitation methods are yet highly significant and examination of purification procedures for many proteins shows that perhaps 80% include at least one step of precipitation with ammonium sulfate.

2.7.1.2 Theory and limits of theory

In a discussion of precipitation of proteins, it is customary to begin with the several theories of precipitation and then proceed to a very prosaic description of general methods. One soon learns that the theory is useful only in a broad sense, and that specific procedures applicable to all proteins or even to classed of proteins cannot be given because the subject is almost embarrassingly empirical. Having said this, knowledge of the theory does provide one with a general understanding of what one is doing and importantly allows an investigator to mark out limits of use of the various agents and conditions being employed. It also sets out precautions to safeguard the structural integrity of particular kinds of proteins and any associated biological activities.

The following discussion encompassed theoretical considerations presented in several reviews. The major forces within a polypeptide chain that drive a protein to a stable conformation at state of minimum energy are ion-ion, ion-dipole (including hydrogen bonds), dipole-dipole and hydrophobic interactions. The structures of proteins in a tissue, whether in solution or associated with insoluble fibers, are influenced by the nature of the aqueous medium as determined by the kinds of other molecules and ions present. Water itself will compete with dipolar interactions in the protein, as many other dipolar substances in the medium. Ions in the medium may competitively disrupt ionic interactions in the polypeptide. Hydrophobic substances in the medium (called lipophilic by organic chemists) may seek out hydrophobic centers in the protein, thus reorganizing the water around the protein. The other macromolecules present may enter into similar interactions with the

protein. When the composition of the medium remains constant, at equilibrium the protein arrives at a stable structure that derives from the sum of all the interactions modified by occurrence of covalent cross-links such as disulfide bonds, if any. In many proteins, both of the globular and fibrous (usually coiled coil) kinds, the "interior" of the structure is shielded from the aqueous medium by a concentration of hydrophobic amino acid residues. The "exterior" or "surface" of the structure interacts with the medium through side chains of polar and ionic amino acid residues clustered or appropriately distributed on the polypeptide. Small patches of hydrophobic groups may also be located on the surface and cause local exclusion-organization of water; however, the bulk of water will hydrate the remainder of the surface through formation of ion-dipole and dipole interactions either ionic or polar groups of the protein. In all cases, exposed parts of the polypeptide backbone ca interact with water, so that even a protein such as elastin, made up primarily of hydrophobic residues, is highly hydrated.

Perturbations of the solvent-protein interactions ca caused transitions in the structure by disrupting the "old" interactions and promoting formation of new ones. If the new set of interactions seeks its own level of minimum energy in which the "interior" is no longer shielded from solvent, it will probably be expressed as a transition to a condition in which the interior is now unfolded.

Perturbations that can cause various conformational transitions include the following. A rise in temperature can weaken the strength of dipolar interaction such as hydrogen bonds and can favor formation of hydrophobic interaction. A decrease in temperature could cause the reverse. Quantitative aspects of the resultant transitions in structure depend on the total numbers of specific kinds of interactions and variation in energies among individual interactions of the same kind. A decrease of pH can cause protonation of certain groups on the surface of the protein can be achieved at which a given protein exhibits no net charge because the number of positively and negatively charged groups are equal, that is, the isoelectric point has been reached. At the pH the surface of the protein will be least solvated (hydrated), and the protein, in nondenatured form, usually ha minimum solubility. It is to be emphasized that minimum solubility and insolubility are no necessarily equivalent and some protein to precipitate. The other perturbation could be introduced to cause

the protein to precipitate. The other perturbation could be addition of a large amount of a salt like NaCl, Na_2SO_4, or $(NH_4)_2SO_4$ or of a water-miscible organic solvent like ethanol or acetone.

Sal in solution at low ionic strength relative to that of isotonic saline may represent a perturbation that can cause certain proteins to precipitate from solution. Such a protein probably was originally maintained in solution in the tissue because it was “salted in” at the ionic strength of the medium. Once a protein of this kind is precipitated it can once again be salted in by solutions of suitable ionic strength.

On the other hand, salts present in very high concentrations with ionic strength much greater than that of tissue media will cause the precipitation of many proteins. Precipitation occurs by neutralization of surface charges by the salt, by reducing the chemical activity of the protein and by diminishing the effective concentration of the water. This is called “salting out” of proteins. The concentration of any salt necessary to cause precipitation of a particular protein is related to the number and distribution of charges and of nonionic polar groups on the surface of the protein and to the number and distribution of hydrophobic residues exposed and rendered dominant as the charges, are neutralized. Of course, the size and shape of the protein contribute to the relative ease of precipitability. All these factors form the basis of salt fractionation of protein mixtures.

2.7.2 Heat treatment and denaturalized precipitation as adjunctive methods in purification of enzymes

Because of the great diversity of structure among proteins it follows that enzymes will exhibit a wide range of stabilities to perturbations in heat and pH. Thus, some proteins such as adenylate kinase and trypsin and certain enzymes of thermophilic microorganisms are relatively heat stable in comparison with the majority of other enzymes. Again, while most enzymes and other proteins undergo denaturalized changes in conformation when exposed to pH values below pH 5 and above pH 10, some proteins have isoelectric points in the alkaline range (e.g., cytochrome c and certain lysozymes), while others have isoelectric points in the acid range (e.g., pepsin, albumin, orosomucoid). In seeking to purify a specific protein

from a mixture of different proteins, the experimenter therefore has the option to use heat and pH to denature and precipitate the unwanted proteins if the conditions used do not affect the desired protein. Such a step can be a valuable subtractive adjunct in the purification procedure. A classic historical example of the use of pH to effect a gross purification was the preparation of a so-called “pH 5 fraction” of tissue homogenates to obtain a soluble extract containing the enzymes of amino acid activation and transfer to tRNA.

2.7.3 Ion-exchange chromatography

2.7.3.1 Net surface charge and pH

IEX separates molecules on the basis of differences in their net surface charge. Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution. The charged groups within a molecule that contribute to the net surface charge possess different pKa values depending on their structure and chemical microenvironment. Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, their net surface charge will change gradually as the pH of the environment changes i.e. proteins are amphoteric. Each protein has its own unique net charge versus pH relationship which can be visualized as a titration curve. This curve reflects how the overall net charge of the protein changes according to the pH of the surroundings. Figure 3 illustrates several theoretical protein titration curves (these curves can be generated using a combination of isoelectric focusing and electrophoresis, but with modern solutions for rapid method development, actual titration curves are rarely used).

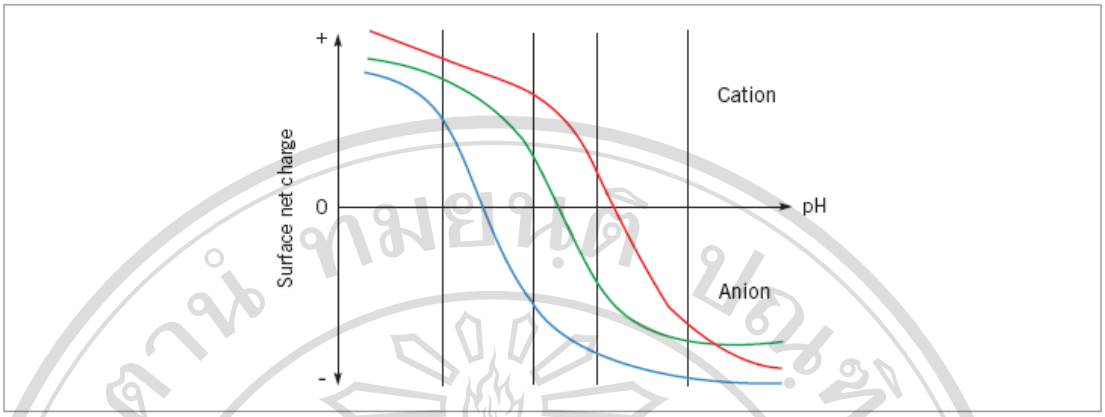

Figure 3 Theoretical protein titration curves, showing how net surface charge varies with pH (Amersham Bioscience, 2002)

IEX chromatography takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein. In an IEX separation reversible interactions between charged molecules and oppositely charged IEX media are controlled in order to favor binding or elution of specific molecules and achieve separation. A protein that has no net charge at a pH equivalent to its isoelectric point (pI) will not interact with a charged medium. However, at a pH above its isoelectric point, a protein will bind to a positively charged medium or anion exchanger and, at a pH below its pI, a protein will behind to a negatively charged medium or cation exchanger. In addition to the ion exchange interaction, other types of binding may occur, but these effects are very small and mainly due to van der Waals forces and non-polar interactions (Amersham Bioscience, 2002).

2.7.3.2 Steps in an IEX separation

An IEX medium comprises a matrix of spherical particles substituted with ionic groups that are negatively (cationic) or positively (anionic) charged. The matrix is usually porous to give a high internal surface area. The medium is packed into a column to form a packed bed. The bed is then equilibrated with buffer which fills the pores of the matrix and the space in between the particles. The pH and ionic strength of the equilibration buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind. The proteins which bind are effectively concentrated onto the column while

proteins that do not have the correct surface charge pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample being loaded.

The condition of the sample is very important in order to achieve the most effective high resolution or group separations and make the most of the high loading capacity. Ideally, samples should be in the same conditions as the start buffer. When all sample has been loaded and the column washed so that all non-binding proteins have passed through the column (i.e. the UV signal has returned to baseline), conditions are altered in order to elute the bound proteins. Most frequently, proteins are eluted by increasing the ionic strength (salt concentration) of the buffer or, occasionally, by changing the pH. As ionic strength increases, the salt ions (typically Na^+ or Cl^-) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher net charge of the protein, the higher ionic strength is needed for elution. By controlling changes in ionic strength using different forms of gradient, proteins are eluted differentially in a purified, concentrated form. A wash step in very high ionic strength buffer removes most tightly bound proteins at the end of an elution. The column is then re-equilibrated in start buffer before applying more samples in the next run. Alternatively, conditions can be chosen to maximize the binding of contaminants and allow the target protein(s) to pass through the column thus removing contaminants (Amersham Bioscience, 2002).

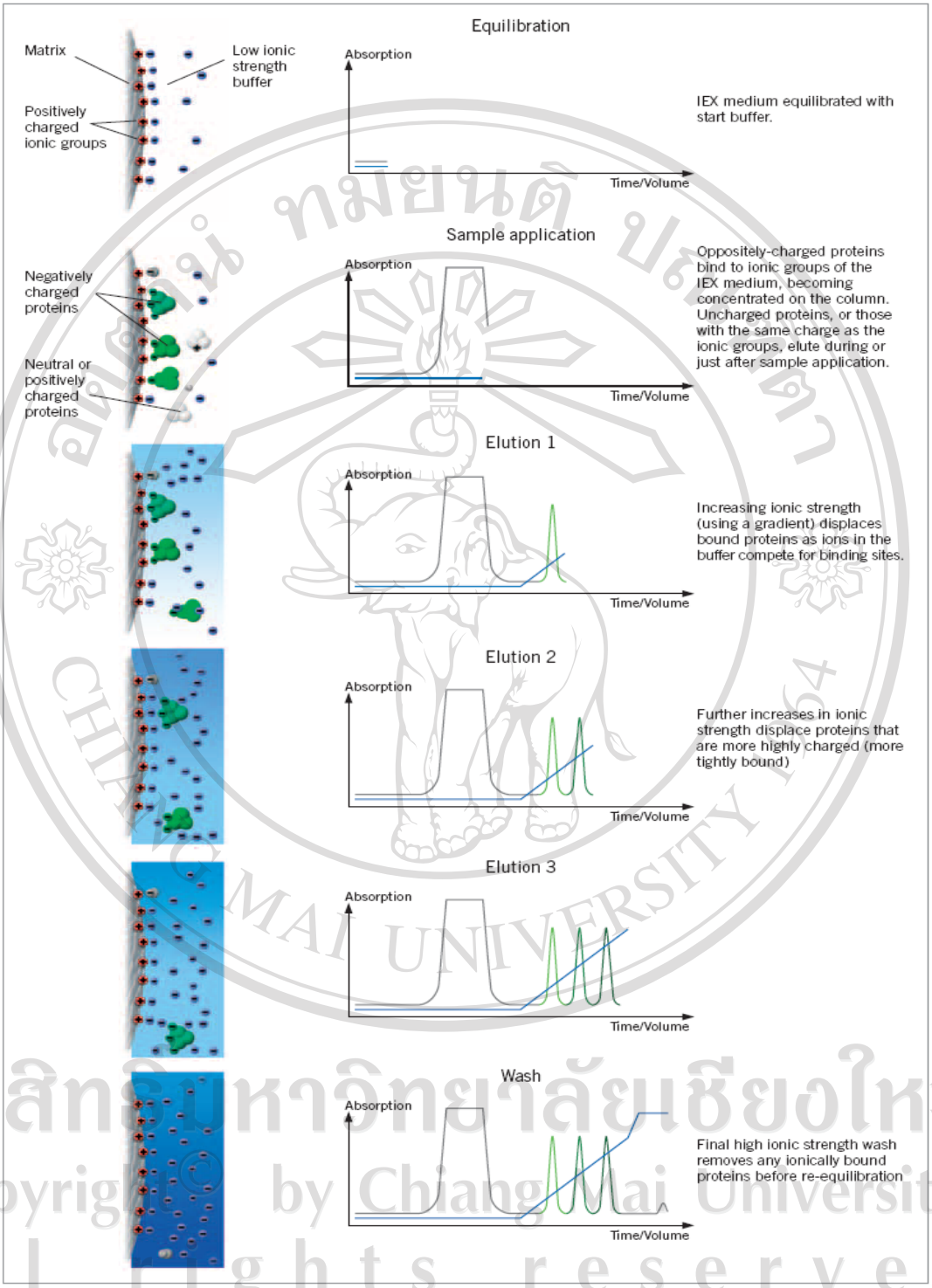

Figure 4 Illustration of the separation process by ion-exchange chromatography (Amersham Bioscience, 2002)

2.7.4 Gel filtration chromatography

Gel filtration is performed using porous beads as the chromatographic support. A column constructed from such beads will have two measurable liquid volumes, the external volume, consisting of the liquid between the beads and the internal volume, consisting of the liquid within the pores of the beads. Large molecules will equilibrate only with both the external and internal volumes. A mixture of proteins is applied in a discrete volume or zone at the top of a gel filtration column and allowed to percolate through the column. The large protein molecules are excluded from the internal volume and therefore emerge first from the column while the smaller protein molecules, which can access the internal volume, emerge later.

The dimensions important to gel filtration are the diameter of the pores that access the internal volume and the hydrodynamic diameter of the protein molecule. The latter is defined as the diameter of the spherical volume created by a protein as it rapidly tumbles in solution. Proteins whose hydrodynamic diameter is small relative to the average pore diameter of beads will access all of the internal volume and are described as being included in the gel matrix. Proteins whose hydrodynamic diameter is comparable to the average pore diameter will access some but not all of the internal volume and are described as being fractionally excluded. Proteins whose hydrodynamic diameter is large relative to the average pore diameter will be unable o access the internal volume and are described as being excluded.

This conceptualization has led to the gradual renaming of gel filtration as size-exclusion chromatography. The order of elution of a mixture of proteins from a size exclusion column will then be the inverse of their hydrodynamic diameters. If all the proteins in a mixture are known, or can be assumed to have the same shape, then the order of elution will be the inverse of their molecular weights. This discussion will treat protein dimensions in terms of molecular weight since common usage assumes that protein mixtures contain only globular proteins. However, the reader should bear in mind that hydrodynamic volume is the operative protein dimension and that an asymmetrical protein will appear to elute with an abnormally high molecular weight compared with globular proteins of similar molecular weight (Amersham Bioscience, 2002).

2.8 Biochemistry and characterization of phytase

The physicochemical characteristics and catalytic properties of phytase from each source were summarized shown in Table 6. The estimated molecular weight was among 35 to 100 kDa depended on the source of origin and usually active within pH range of 4.5-6.0.

Ullah and Gibson (1987) and Ullah and Cummis (1987) reported that phytases (phyA and phyB) from *A. niger* NRRL 3135 were produced increasingly after growing the strain under phosphate starvation condition in starch medium. Ion exchange chromatography and chromatofocusing were used for phytases purification. It was found that optimum pH of phytases was 6.0 and the molecular weight was estimated of 85, 65 kDa for phyA and phyB acid phosphatase, respectively. Casey and Walsh (2003) purified and characterized thermostable extracellular phytase from *A. niger* ATCC 9142 by ion-exchange and gel filtration chromatography and chromatofocusing technique. The enzyme was optimally active at 65°C, pH 5.0 with broad substrate specificity and still stable until 80°C. Golevan *et al.* (2000) purified *E. coli* phytase which had 45 kDa of molecular weight and further separated into two isoforms of identical size with pI of 6.5 and 6.3 by chromatofocusing. The isforms showed similar optimum temperature and pH of 60°C and 4.5, respectively. Tambe *et al.* (1994) isolated two isoforms of phytase from *Klebsiella aerogenes* which had different molecular weight, pI, K_m, thermostability, pH and temperature optima. It was also found that the enzyme had such a small molecular weight as 10 to 13 kDa. Vats and Banerjee (2004) reported that general phytases from bacterial sources have optimum pH in neutral to alkaline range while in fungi, optimum pH range is 2.5 to 6.0 and the stability of phytase decreases dramatically above pH 7.5 and below pH 8.0. This wide range of different in pH optima could be due to the variation in molecular conformation or stero-specificity of the protein from different sources. Phytase in general shows high activity in temperature is mostly between 45 and 60°C.

Table 6 Physicochemical and kinetic properties of phytase

Sources	Molecular weight (kDa)	Optimum		Substrate specificity	K_{cat} (s^{-1})	K_M (mM)	References
		pH	Temp.(°C)				
Bacteria							
Bacillus sp. DS11	44	7.0	70	Specific	-	0.55	Kim *et al.* (1998a,b)
B. subtilis	36-38	6.0-6.5	60	Specific	5.5	0.5	Kerovuo *et al.* (1998) and Stock *et al.* (2003)
B. amyloliquefaciens (168 phyA, phyL)	44, 47	5.0, 5.1	55, 65	Specific	-	-	Tye *et al.* (2002)
Enterobacter sp.	-	7.0-7.5	50	-	-	0.70	Yoon *et al.* (1996)
Escherichia coli	42	4.5	60	Specific	6209	0.13	Ostanin *et al.* (1993)
Klebsilla terrigena	40	5.0	58	-	-	-	Greiner *et al.* (1997)
Psuedomonas syringae	45	5.5	40	Specific	-	0.38	Cho *et al.* (2003)
Citrobacter braakii	47	4.0	50	-	-	0.46	Kim *et al.* (2003)
Yeast							
Arxula adeninivornas	-	4.5	75	Specific		0.23	Sano *et al.* (1999)
Schwanniomy ces castellii	490	-	77	-	-	0.038	Segueilha *et al.* (1992)
Saccharomyces cerevisiae	120	2.0-2.5, 5.0-5.5	55-60	-	-	-	Han *et al.* (1999a)

Table 6 Physicochemical and kinetic properties of phytase (continued)

Sources	Molecular weight (kDa)	Optimum		Substrate specificity	K_{cat} (S^{-1})	K_M (mM)	References
		pH	Temp. (°C)				
Yeast (cont.)							
Pichia pastoris	95	2.5-5.5	60	-	-	-	Han *et al*. (1999b)
Fungi							
Aspergillus ficuum (phyA)	85	2.5, 5.0	58	Specific	348	0.03	Ullah and Gibson (1987)
A. ficuum (phyB)	68	2.5	63	Broad	628	0.103	Ehrlich *et al*. (1993) and Ullah and Cummin (1987)
A. oryzae	120	5.5	50	Broad	-	0.33	Shimizu (1993)
A. niger SK-37	60	2.5, 5.5	50	Specific	-	0.019	Nagashima *et al*. (1999)
Peniophora lycii (phyA)	72	4.0-4.5	50-55	Specific	-	-	Lassen *et al*. (2001)
Plants							
Canola seed	-	5.2	50	Broad	-	-	Ebune *et al*. (1995) and Nair and Duvnjak (1990)
Zea mays	60	4.5-4.8	55	Broad	-	0.117	Gibson and Ullah (1988)

Source: Vats and Banerjee (2004)