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The Use of Phytate-Degrading Enzyme Extracted From Germinated Legumes in Food Processing

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Keywords:

Legume seeds, soaking, germination, phytase activity, phytate phosphorous, inorganic phosphorous.

Abstract

The aim of this study was an attempt to use phytase enzyme extracted from plant sources such as germinated legumes to reduce phytic acid content in faba bean, lentil, pea, chickpea and kidney bean during soaking and germination processes. Legume seeds are a good source of phosphorus compounds. Phytic acid contents in raw legume seeds such as faba bean, lentil, pea, chickpea and kidney bean were 1.10, 0.95, 0.86, 0.73 and 1.20%, respectively. The highest phytase activity was found in the germinated faba bean for 144 hrs with partial purification by acetone 80%. Soaking process of legumes with ordinary water without addition of phytase extract led to minimal reduction of phytate content in all tested legumes. The reduction rate of phytate was 6 and 12% of its initial content in raw seeds after 8 and 12 hours, respectively. The hydrolysis of phytate was enhanced with increasing ratio of added phytase extract to water (45 : 55) during soaking for 12 hrs. A significant reduction in phytate content during the germination process as a result of the phytase extract addition was found. The loss rates of phytate were 47.9, 59.2, 70.4, 75.8 and 90.3% of its content in raw seeds after 24, 48, 72, 96 and 120 hrs respectively. The high loss of phytate content during soaking and germination was due to the addition of high activity enzyme extract as well as the endogenous enzyme effects. As a result of phytate hydrolysis during germination, the inorganic phosphorus(Pi) in all germinated legumes was librated in a large amounts with addition exogenous phytase compared to the phosphorus released by the native phytase.

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INTRODUCTION

phytate-degrating enzymes [phytases] catalyze the hydrolysis of phytate (*myo*-inositol hexa-phosphate, IP6), the major storage form of phosphorus in plant kingdom. Phytases belong to a special group of phosphatases, that are chemically known as *myo*-inositol (1.2.3.4.5.6) hexakisphosphate phosphohydrolase, and catalyze the sequential release of phosphate from phytate **(Frias *et al.*, 2003; Trann *et al.*, 2011).** Phytase not only releases the phosphorus from plant-based diets, but also makes available calcium, magnesium, protein and lipid. Thus, by releasing bound phosphorus in feed ingredients of for bone growth and protects the environment against

phosphorus pollution **(Baruah *et al.*, 2007; da Luz *et al.*, 2012).** Phytases can also be broadly categorized into two major classes based on their optimum pH: the histidine acid phosphatase and alkaline phytase. The first showed the optimum activity at pH around 5.0, whilst the second are more pronounced at pH near to 8.0 **(Baruah *et al.*, 2007).** Phytase are widely distributed in nature in plants **(Konietzny *et al.*, 1995)** and microorganisms **(Mullaney and Ullah, 2003; Osman *et al.*, 2012).** Phytic acid is an inhibitor of mineral absorption because the negative charges of o\phosphate groups from insoluble salts upon interaction with di- and tri-valent cation such as Ca, Fe, Mg and Zn. It is also the principal storage form of phosphate in plant seeds **(Enujiugha, 2005; Luo and Xie 2012).** According to **Sandberg (2002),** mineral content of legumes is generally high, but the bioavailability is poor due to the presence of phytate, which is a main inhibitor for Fe and Zn absorption. Phytase is responsible for phytate degradation thereby leading to increased bioavailability of the affected elements in food. Sprouting is the practice of soaking and leaving seeds until they germinate and begin to sprout. This practice is reported to be associated with improvements in the nutritive value of seeds **(Greiner *et al.*, 2001; Zanabria *et al.*, 2006 and Kumar *et al.*, 2010).** At the same time, there are indications that germination is effective in reducing phytic acid **(Kalpanadevi and Mohan 2013).** In case of white kidney beans, Faba beans and chickpeas; sprouting improved the protein/ amino acid digestibility by decreasing antinutritional factors and increasing the true/ apparent protein/ amino acid digestibility **(Rubio *et al.*, 2002).** Legumes provide a large amount of protein, carbohydrates, dietary fiber, minerals and water-soluble vitamins in human diets. In some areas of the world, where the predominant diet pattern is vegetarian or animal meat is available in only small amounts, legumes provide the major source of proteins. Therefore, legumes can be considered as foods with health benefits, but their phytate contents can limit the availability of minerals. Whole meal cereals and legumes products have a high mineral content, but they also contain considerable amounts of phytic acid which chelates with minerals such as calcium **(Ekholm et al., 2003),** iron **(Gerald, 2002),** zinc **(Lönnerdal, 2000),** magnesium **(Coudray *et al.*, 2001)** and copper **(Lopez *et al.*, 2002).** Thus, phytases have an important application in human nutrition both for degradation of phytate during food processing and in the gastrointestinal tract. However, the capability to dephosphorylate phytate differs greatly among different plant and microbial species due to differences in their intrinsic phytate-degrading activities. Phytate hydrolysis can occur during food preparation and production, either by phytase from plants or by microorganisms. Foods processing which increase the activity of native enzymes are soaking, cooking, malting, germination, baking and fermentation technology. Fermentation leads to lowering of pH

as a result of bacterial production of organic acids especially lactic and acetic acid, which is favorable for cereal phytase activity **(Egli *et al.*, 2002).**

The aim of this study was an attempt to use phytase enzyme extracted from plant sources such as germinated legumes in food processing. The partially purified enzyme was used during the soaking and germination processes in some common legumes

Materials and Methods

Materials: Faba bean seeds (*Vicia faba cv. Giza 3*) were obtained from Agronomy Research Institute (Shandaweel Agricultural Research Center, Sohag, Egypt). Seeds of lentil (*Lens eulimaris cv. Giza 9*), peas (*Pisum sativum var. Alaska*) and chickpea (*Cicerarietinum*) were obtained from Vegetable Research Institute (Agricultural Research Center, Giza - Egypt).

Chemicals: Dodecasodium phytate, acetic acid, sodium acetate and calcium chloride were obtained from Sigma (Germany). Imidazol($C_3H_4N_2$) was purchased from Alderich (Germany).

Method:

Soaking and germination: Legume seeds were cleaned, washed and soaking for 12 hrs in tap water at room temperature (25±2^{0}C) as reported by **Abdel-Gawad (1993).** After soaking a part of soaked samples was used for phytase activity assay. The other part was used for germination in the dark till 144 hrs (for Faba bean and chickpea) and 120 hrs for lentil and pea as described by **Abdel Gawad (1991) and Oraby, 2005).** Seeds were sprayed every 12 hrs by sterilized water as neede, and the surface was sterilized by resoaking in 1% sodium hypocholite solution for 10 min, Produced seedlings were dried at 60^{0}C for 48 hrs and ground in an electric grinder to pass through a 100 mesh (0.15 mm) sieve and stored in closed bottles in a refrigerator at 5^{0}C±1 until analysis.

Extraction of phytase: The crude enzyme was extracted described by **Abdel-Gawad and Hamada (2000)** by stirring samle in 0.1 M acetate buffer, pH 5.2, (using 1 flour : 10 buffer, W/V) at 5-10^{0}C for 30 min, then centrifuged for 20 min at 4200 *xg* and finally filtering the supernatant through four layers of filter cloth. The obtained filtrate was mixed with cold acetone to precipitate the enzyme. The produced precipitate was re-dissolved in acetate buffer (pH 5.2), dialyzed over night against the same buffer and centrifuged as mentioned above. The obtained supernatant was the partial purified phytase then using in food processing.

Moisture content: Moisture content of soaked and germinated legume seed samples which previously dried (at 60^{0}C, for 48 hrs) were finally performed at 105^{0}C for 3 hrs according to **A.OA.C. (1990)** methods.

Determination of phytase activity: The activity of partial purified phytase was measured as described by **Lolas and Markakis (1977).** The reaction

mixture contained 4.0 ml 0.1M acetate and 0.1M imidazol-Hcl buffers (pH optimal 5.0 for lentil, pea, chickpea) and 5.2 for faba been) 0.2 ml of 2 mM dodecasodium phytate and 0.5 ml enzyme extract. After incubation at optimum temperatures (50^0C for faba been, lentil, chickpea and 45^0C for pea) for 60 min as described by **Oraby (2005).** The reaction was stopped by addition of 0.5 ml 10% TCA. Inorganic phosphorus liberated by phytase determined by measuring the absorbance at 680 nm after 10 min as stated by **Chen *et al.*, (1956).** Potassium dihydrogen phosphate was used as standard. The activity of phytase was expressed as micromole (µM) inorganic phosphorus (Pi) liberated in one minute per 1g sample.

Determination of phosphorus compounds: Total phosphorus (TP), inorganic phosphorus (Pi) and phytate phosphorus (PP) were measured. The digestion of the samples before the determination of of TP was carried out according to the method described by **Tangkonchitr *et al.*, (1981a).** The ammonium molybdate colorimetric method for phosphorus determination was used in this study **(Jackson, 1973).** The native inorganic phosphorus present in the samples was extracted according to the procedure of **Tangkonchitr *et al.*, (1981b)** and determined as described method of **Jackson (1973).** Phytate phosphorus was extracted as below described in determination of phytic acid and digested using mixture of 1 ml sulfuric acid and 6 ml nitric acid to release the phosphorus as described in **A.O.A.C. (1990).** The phytate phosphorus was measured colorimetrically as above described. Phytic acid (*myo*-inositol hexaphosphate) was calculated by multiplying the value of phytate phosphorus × 3.546 factor.

Results and Discussion

Phytic acid and phosphorus compounds in raw legume seeds. Phytic acid (AP), phytate phosphorus (PP), inorganic phosphorus (Pi) and total phosphorus(TP) were determined in legume seeds as shown in Table (1). Faba bean and kidney bean had higher phosphorus compounds content than those found in lentil, pea and chickpea. Phytate phosphorus as reserve of phosphorus in the legume seeds and represented 40.5-63.5% of total phosphorus in legumes under investigation. The results in Table (1) indicated that, the phytic acid contents in raw legumes, faba bean, lentil, pea, chickpea and kidney bean were 1.10, 0.95, 0.86, 0.73 and 1.20%, respectively. Phytate phosphorus in raw legume seeds were; 310.9, 268.5, 241.2, 206.1 and 324.7 mg/100g on dry weight bases for raw faba bean, lentil, pea, chickpea and kidney bean, respectively. Data in the same Table also showed that, the contents of inorganic phosphorus were; 74.9, 62.9, 55.3, 55.0 and 60.7 mg/100g on dry weight bases in faba bean, lentil, pea, chickpea and kidney bean, respectively. The values of total phosphorus in faba bean and kidney bean were higher than that in the other legumes, while chickpea seeds contained the lowest value. These results are in the line with those

reported by Sorour (2002), who stated that, phytic acid in cowpea and faba bean were 9.47 and 11.45 (m/g), respectively. Horner et al (2005) mentioned that, the total phytate (Ins P6) of 35 soybean cultivars varied from 1.01 to 1.82 (g/100g). Salem (2007) found that, the raw sample contained amounts of phytic acid were 1.30 and 1.10% in cowpea and field bean, respectively. Porres *et al.*, (2004) reported that the contents of total phosphorus and phytate phosphorus in raw lentil flour were 387.6 and 270 mg/100g dry sample, respectively.

Table (1): Phytic acid and phosphorus compounds contents in some raw legumes.

Samples	Phytic acid* (mg/100g)	Phytate phosphorus (mg/100g)	Inorganic phosphorus (mg/100g)	Total phosphorus (mg/100g)	Phytate phosphorus as % of TP
Faba bean	1103.2	310.9	74.9	650.1	47.8
Lentil	952.7	268.5	62.9	558.6	48.0
Pea	855.9	241.2	55.3	595.8	40.5
Chickpea	731.3	206.1	55.0	324.7	63.5
Kidney bean	1152.2	324.7	60.7	635.3	51.1

*** Calculated from phytate phosphorus content.**

Phytase activity in raw, soaked and germinated legumes: Based on previous studies (**Abdel-Gawad *et al.*, (2013)**, it was found that the highest activity of phytase enzyme was at 144 hrs of germination for faba bean and pea while, the highest activity for lentil, chickpeas and kidney bean was at 120 hrs. Phytase was determined in raw, soaked and germinated faba bean, lentil, pea, chickpea and kidney bean verities as micromole (µM) inorganic phosphorus liberated from substrates in one minute per gram of dry samples (Figure 1). The results revealed that there are an increase in phytase activity with purification of extracted enzyme (by acetone 80%) in all legume samples. The highest phytase activity was found in germinated faba bean for 144 h with partial purification by acetone 80%. Phytase activity of germinated faba bean, lentil, pea, chickpea and kidney bean were; 17.62, 12.52, 16.28, 11.38 and 14.18 µM/g/min, respectively. **Greiner *et al.*, (2001),** reported that phytase activity in dry faba bean seeds

accounted for 0.06 ± 0.01 Unit/g of seed. Which increased to maximum activity after 4 days of germination (1.35 ± 0.02 Unit/g of seeds). The results are in agreement with the investigation of **Abdel-Gawad *et al.*, (2013)** who reported that faba bean and pea showed high increases in their phytase activity by 10.3 and 8.5 folds after 144-hrs germination, whereas lentil and kidney bean by 7.5 and 7.7-folds after 120-hrs germination, after that the enzyme activity decreased with prolonging time of germination.

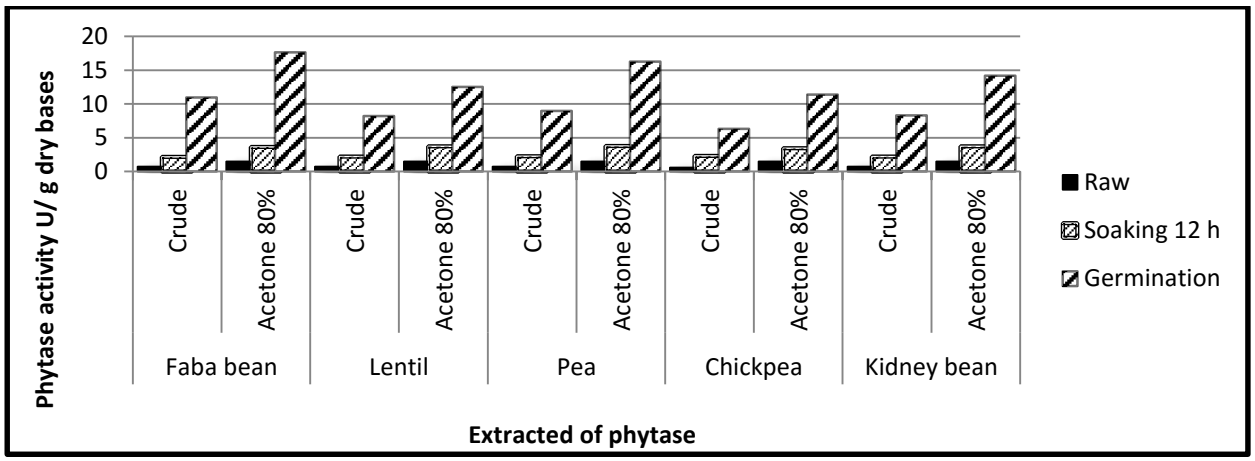

Figure (1): Phytase activity extracted from some legumes.

***Effect of phytase extract addition on phytate degradation during soaking of legumes*.** Because the faba bean sprouts contain high phytase activity compared to other legumes, therefore, the phytase extracted from faba bean can be used to reduce phytic acid during soaking and germination of legume seeds. The results illustrated in Figure (2) shows the effect of addition the extracted phytase from germinated faba bean on reduction of phytate during soaking of faba bean. The effect of gradual increasing amounts of added phytase extract ratio : water (45 : 55) on phytate degradation during soaking of faba bean were investigated. Soaking process of legumes with ordinary water without addition of phytase extract led to slightly reduced the phytate content in all tested legumes. The decrease in phytate content was minimal after 8 and 12 hours of soaking. The reduction rate of phytate was 6 and 12% after 8 and 12 hours of its initial content in raw seeds, respectively. The data indicated that, the hydrolysis of phytate was enhanced with increasing the ratio of added phytase extract to water (45 : 55) during soaking for 12 h, phytate contents in soaked faba bean decreased to 64.2 and 79.7% of its initial values periods 8 and 12 h, respectively. The significant decrease of phytate content during soaking of faba bean may be due to the phytase activity increased in the added extract to soaking water. **Fernández *et al.*, (1997)** reported that, soaking of faba bean in distilled water, acid solution or

basic solution decreased phytic acid by 30%.

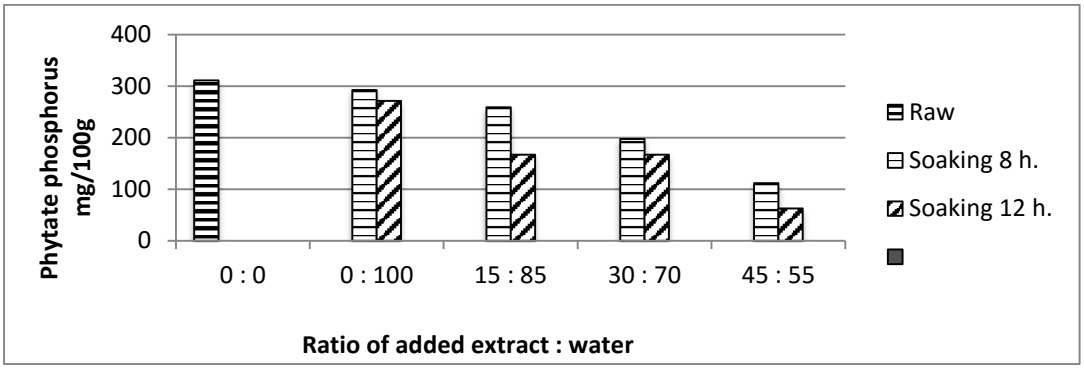

Figure (2): Effect of addition extracted phytase from germinated faba bean on phytate reduction during soaking faba bean.

The data showed that, when the high activity enzyme extract is added to the soaking water during the process of soaking lentil, chick pea and kidney bean seeds (Figur 3, 4, and 5), a gradual decrease of phytic acid occurs by increasing the added extract. By adding 45% enzyme extract of the soaking water and soaking for 12 hours, the phytate content decreases by 78.4%, 79.5 and 79.6% of its initial values in lentil, chick pea and kidney bean, respectively. The results indicated that, inorganic phosphorus (Pi) gradually increased with the addition of added extract increasing and reached to 274.3, 218.1 and 317.7 mg/100g after 12 hours of soaking in lentil, chick pea and kidney bean, respectively. The increasing of inorganic phosphorus during soaking may be due to the hydrolysis of phytic acid as a result of increased phytase activity as well as degradation of phytate to inorganic phosphorus. **Ali, (2008).** The results also indicated that, legume seeds represent a rich source of phosphorus. Cowpea, faba bean and pea had higher phosphorus content (584.4–751.4 mg/100g sample) than that found in lentil and chickpea (309.9–549.8 mg/100g sample). Inorganic phosphorus content of raw legume seeds ranged from 52.2 to 73.1 mg/100g sample. **Kumar *et al.*, (2010)** reported that, a considerable amount of phytate is removed into the water. On the other hand, process may be enhances the action of naturally occurring phytase in soaked legumes.

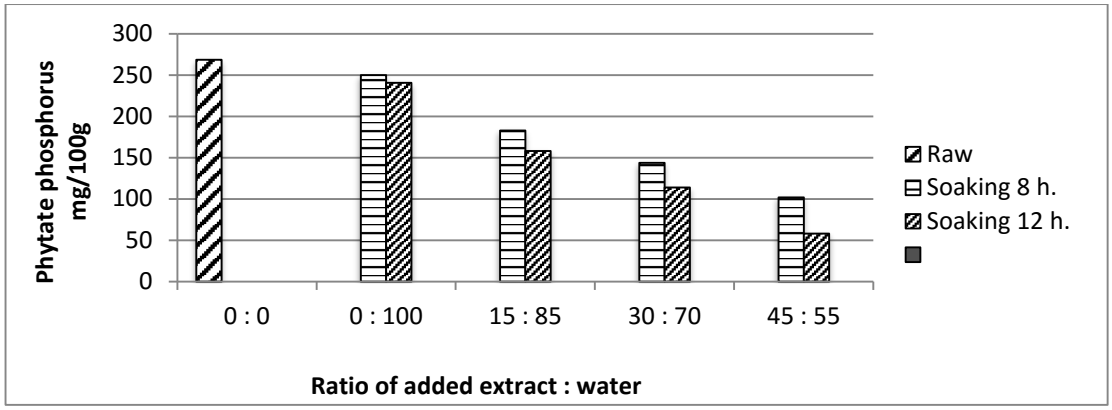

Figure (3): Effect of addition extracted phytase from germinated faba bean on phytate reduction during soaking lentil.

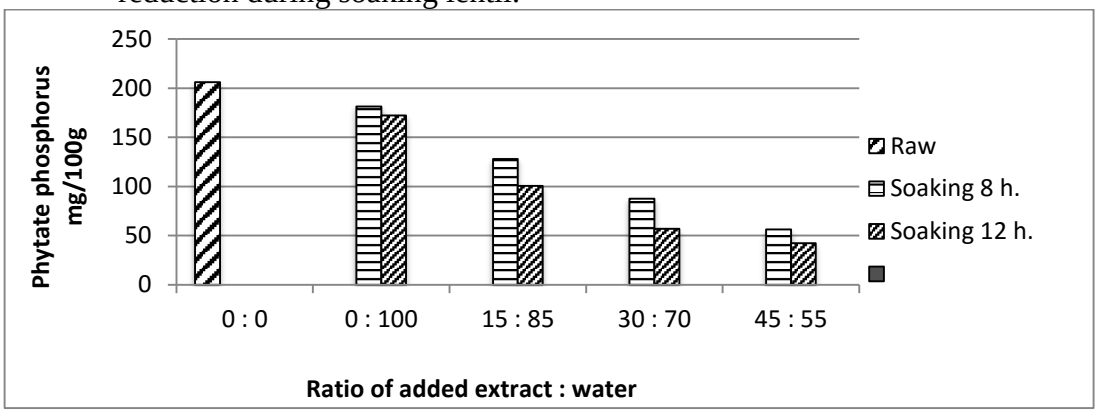

Figure (4): Effect of addition extracted phytase from germinated faba bean on phytate reduction during soaking chickpea.

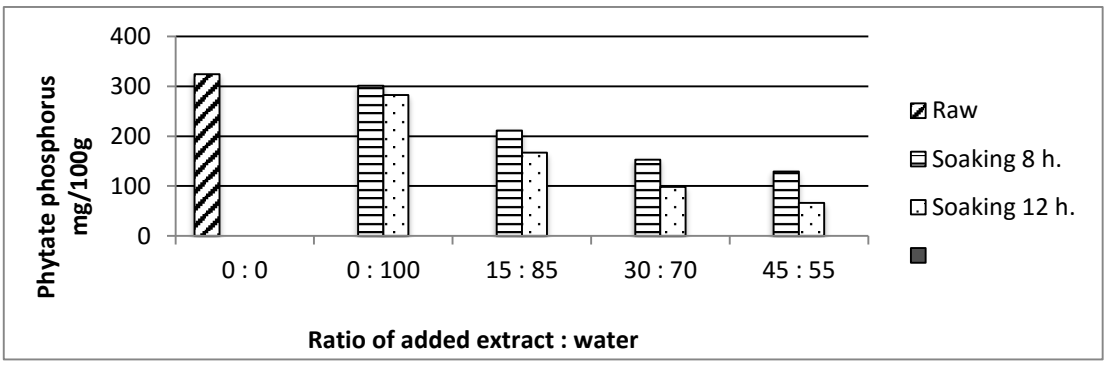

Figure (5): Effect of addition extracted phytase from germinated faba bean on phytate reduction during soaking kidney bean.

Effect of addition phytase extract on phytate reduction during legume germination. As shown in Table (2), the addition of phytase extracted from germinated faba bean to the seeds during soaking and germination was investigated. The results indicated that, the process of germination without addition of extracted phytase reduced the phytate contents of the germinated faba bean by 40.8, 52.1, 65.7, 70.3 and 75.4% after 24, 48, 72, 96 and 120 hrs,

respectively, of it initial content in the raw seeds. The decreasing in phytate content is due to the activity of the endogenous phytase enzyme during the germination process. The results in the same Table indicated that, a highly reduction in phytate content during the germination process was found as a result of the addition of the phytase extract. The loss rate of phytate was 47.9, 59.2, 70.4, 75.8 and 90.3% of its content in the raw seeds after 24, 48, 72, 96 and 120 hrs, respectively. The obtained results showed that, the decrease in phytate content with addition of phytase extract in the other tested legume seeds after 120 hrs of germination were: 89.1, 85.4, 87.7 and 86.4% of its original values of lentil, pea, chickpea and kidney bean, respectively(data not shown). The high loss of phytate content during germination was due to the addition of faba bean extracted enzyme, in addition to the endogenous enzyme. As a result of the hydrolysis of phytate during germination, the inorganic phosphorus(Pi) in all germinated legumes is librated in a large amounts as a result of the addition of the exogenous phytase compared to the phosphorus released by the endogenous phytase. The decrease in phytate phosphorus and increase in inorganic phosphorus during germination is a result to increase of phytase activity which degraded the inositol hexaphosphate **(Abdel-Gawad, 1991). Ghavidel and Prakash (2007)** studied the effect of germination for 24 hrs on phytic acid content of green gram, cowpea, lentil and chickpea and stated that phytic acid reduced by 18-21% in tested legume seeds after germination.

Table (2): Effect of addition phytase extract on phytate degradation during germination

Period of germination (h)	Germination with out phytase extract			Germination with phytase extract		
	pp[a]	Pi[b]	pp reduction (%)	pp	Pi	pp reduction (%)
	(mg/100g dry weight)			(mg/100g dry weight)		
0	310.9	74.9	---	310.9	74.9	---
24	184.2	225.3	40.8	162.1	200.6	47.9
48	148.8	256.9	52.1	126.7	236.4	59.2
72	106.7	276.6	65.7	91.9	293.9	70.4
96	92.3	290.4	70.3	75.1	310.9	75.8
120	76.6	306.5	75.4	30.0	356.0	90.3

(a) : Phytate phosphorus. **(b) :** Inorganic phosphorus.

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