Research Article

Determination of Different Inositol Phosphate Forms in Raw, Soaked and Germinating Legumes by Anion-Exchange Chromatography

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Abstract The separation and quantitative determination of phytic acid (inositol hexaphosphate IP_6) and lower inositol (tri-IP_3, tetra-IP_4 and pentaphosphates IP_5) in raw, soaked and germinating faba bean, lentil, chickpea and pea was carried out using an anion exchange column chromatography. The raw faba bean, lentil, chickpea and pea contained higher concentration of IP_6 ranged from 637 to 836.9 mg/100g d.m. The IP_5 was found in raw faba bean and lentil, whereas, IP_3 was detected only in raw chickpea. Soaking for 12 hrs diminished IP_6 and increased IP_5 and inorganic phosphate (P_i) in faba bean and lentil, while in chickpea and pea, the decrease of IP_6 was accompanied by formation of IP_5 and increase of P_i. The germination for 48 hrs caused lowering in IP_6 and increment in IP_5 with formation of IP_4 and IP_3 in faba bean and lentil. Sharp decreases in IP_6 and IP_5 were accompanied by increases in IP_4 and IP_3 in faba bean and lentil after 72-hrs germination. Furthermore, in chickpea and pea showed a large dropping in the contents of IP_6 and IP_5 after 72 hrs germination, in this paper, different legumes with the phytate content and their hydrolysis products will be presented.

Keywords legumes, soaking, germination, forms, inositol phosphates.

1. Introduction

Phytic acid has long been known as an anti-nutritional factor since it reduces bioavailability of several minerals due to its ability to chelate them [1]. Six phosphate groups in the molecule of IP_6 make a strong chelating agent, which binds minerals such as Ca^{+2}, Mg^{+2}, Fe^{+3} and Zn^{+2}. Under gastrointestinal pH conditions, insoluble metal-phytate complexes are formed [2] which make the metal unavailable for absorption from the intestinal tract of animals and humans [3]. Phytic acid is able to complex with proteins also, decreasing protein solubility. Phytates impact on enzyme activity with evidence of a negative effect on key digestive enzymes including pepsin, trypsin, chymotrypsin, amylase and lipase [4] therefore phytic acid reduce the digestibility of protein [5], starch [6], and lipids [7].

At present, growing concern about phytic acid and their hydrolysis products has arisen from the finding that it might have beneficial effects such as antioxidant function, protecting against cancer risk [8]. It has protective effect on neurons in parkinson's disease [9], and it has also potential role and therapeutic interest in metabolic diseases [10]. In addition, some products hydrolysis of inositol hexaphosphates play key biological roles. The major representative of the later is *D-myo*-inositol-1,4,5- triphosphate which is known for its signaling activities and receptor for the major intracellular Ca^{+2}-release channels in cells [11].

During food processing, inositol hexaphosphate can be partially dephosphorylated to produce degradation products by the action of endogenous phytases, which are found in most seeds from higher plants [12]. Deferent processes, such as soaking [13], germination, cooking [14] and fermentation [15] , reduces the content of IP_6 in legumes. During soaking and germination of legumes, phytic acid is enzymatically hydrolyzed by endogenous phytases to lower inositol phosphates such as inositol pentaphosphate (IP_5), inositol tetraphosphate (IP_4), inositol triphosphate (IP_3) and possibly the inositol di- and monophosphates [16]. Only IP_6 and IP_5 have a negative effect on the bioavailability of minerals, the other hydrolytic products formed have a poor capacity to bind minerals, or the complex formed are more soluble [17]. Natural endogenous phytases can be activated during soaking and germination of legumes and hydrolyzed the IP_6 to lower inositol phosphates [12]. Furthermore endogenous plant phytases are of great interest in the production of special isomers of the different lower phosphate esters of *myo*-inositol [18]. Attempts to produce different phosphate esters of *myo*-inositol enzymatically from phytic acid have resulted in mixtures of IP_5, IP_4, IP_3 and IP_2 [19].

Legumes provide a large amount of protein, carbohydrates, dietary fiber, minerals and water-soluble vitamins in human diets. Therefore, legumes can be considered as foods with health benefits, but their phytate contents can limit the availability of minerals [14]. In Egypt, legumes constitute important food stuff and are chief economic sources of proteins in the diets of economically weaker sections of population. Legumes such as faba bean, lentil, chickpea and pea are consumed widely in Egypt; and are also in price inexpensive, but contains certain anti-nutritional factors; one of these is phytic acid. These legumes contains after processing, in addition to IP_6, lower inositol phosphates which have much less affinity or no potential for reducing the bioavailability of minerals and it calculated as phytic acid or anti-nutritional factor by classical absorptiometric methods for estimation of phytate. Therefore, the objectives of this study were undertaken to study the content of different forms of inositol phosphates in Egyptian legumes and investigate the effect of soaking and germination on the changes of inositol hexaphosphate (IP_6) to health-beneficial lower inositol phosphate.

2. Materials and Methods

2.1 Materials:

The seeds of faba bean (*Vicia faba* cv. Miser 1), lentil (*Lens culinaris* cv. Giza 133), chickpea (*Cicer arietinum* cv. Giza 2) and Pea (*Pisum sativum* cv. Master B) were obtained from Vegetable Research Institute (Agricultural Research Center, Giza - Egypt).

2.2 Chemicals:

Ion-exchange resin Dowex 1X2 (200–400 mesh, Cl⁻ form; Sigma, Germany); Dodecasodium phytate (Sigma; Germany) and Inositol-*1,4,5*-triphosphate, IP_3, (gift from Dr. W. Yokoyama, USDA, ARS, Western Regional Research Center).

2.3 Processing

2.3.1 Soaking:

Samples of dry legume seeds were soaked in tap water for 12 hrs at room temperature (25 ±2℃). The seeds to water ratio was 1:4 (W/V). After the end of soaking, the water was drained, then a part of soaked samples was dried at 50℃ in a hot air oven for 48 h and finally ground in an electric grinder to pass through a 100-mesh (0.15 mm) sieve [20], and the other part were used for germination.

2.3.2. Germination:

For the germination of legumes, a part of 12-hrs soaked seeds of each faba bean, lentil, chickpea and pea were surface sterilized by re-soaking in 1% sodium hypochlorite solution for 10 min, then washed with sterilized water and the germination was carried out on a wet cellulose pads in the dark for 48 and 72 hrs at 22 ±2 ℃. The seeds were sprayed every 12 hrs with sterilized water as needed. The seedlings were dried at 60 ℃ for 48 h and then ground in an electric grinder to pass through a 100-mesh (0.15 mm) sieve [21]. The ground samples were kept in closed bottles and stored in a refrigerator at 5 ℃ until analysis.

2.4 Analytical Methods:

Moisture of all raw, soaked and germinating samples were determined according to AOAC [22] standard methods.

2.4.1 Phytic acid Extraction:

The phytic acid extraction was accomplished using 0.5 M HCl in a 1:10 ratio of legume sample to acid [23]. The suspension was shaken mechanically for 2 hr and subsequently centrifuged for 15 min at *8,000 xg*; then the supernatant filtered through a Whatman # 1filter paper.

2.4.2 Standards:

The standards used were inositol-1,4,5-triphosphate (IP_3) and dodecasodium phytate (IP_6). In addition, a mixture of inositol phosphates was prepared by partial hydrolyzing an aqueous solution of dodecasodium phytate (6 mg/ml) by autoclaving at l20 °C for 1 h as the method described by Hara et al [24]. Phosphorus content of dodecasodium phytate was 19.03 % determined after acid hydrolysis [25] by method of Jackson [26].

2.4.3. Separation and analysis of inositol phosphates:

In the ion-exchange method [27], a glass column (1 x 15 cm) was packed with about two gram of Dowex 1X2 (200–400 mesh, Cl⁻ form; Sigma, Germany). Before use, the column was washed first with 1 M HCl to assure chloride saturation of the resin and then washed with distilled water until the eluate was salt-free. 5 ml of phytate extract of legume sample were diluted before being placed on the column so that the total anion concentration was less than 0.05 M (about 1:25). The sample container was rinsed and the column was washed with 30 ml of redistilled water to elute most of inorganic phosphorus. The remaining inorganic phosphate was eluted with 30 ml 0.05 M HCl. Linear gradient elution was performed with 650 ml of 0.0 - 0.7 M HCl (0.8 ml/min). Ten ml fractions were collected. Two ml of each fraction were acid-digested by method of Tangkongchitr et al [25] and followed by determination of total phosphorus by Jackson [26]. On the basis of phosphorus analysis, the fraction of a subsequent identical run were gathered and combined. Five ml of each were mixed with 0.8 ml concentrated HCl to make a solution of about 6 N HCl and then hydrolyzed at 110ºC in sealed ampoules for 48 hrs. The hydrolysate was subjected to inositol determination according to Agranoff et al [28] and to phosphorus assay [26]. The molar ratio, inositol : phosphorus, were allowed the identification of the inositol phosphate type corresponding to each peak.

The dodecasodium phytate and inositol-*1,4,5*-triphosphate were chromatographed on Dowex column with the same technique described above as standards for definition the position of IP_6 and IP_3, respectively. The other low inositol polyphosphates (IP_5, IP_4, IP_2 and PI_1) were obtained by separation of partial hydrolyzed dodecasodium phytate as the same technique described above. The concentrations of different forms of inositol phosphates in legumes (mg per 100 g sample on dry basis) was calculated from the weight ratio of phosphorus atoms per molecule of IP_6 (1: 3.52), IP_5 (1: 3.71), IP_4 (1: 3.99) and IP_3 (1: 4.46).

3. Results and Discussions

3.1. Chromatographic separation of inositol phosphates:

A reference standard of dodecasodium phytate was chromatographed on a column of Dowex 1X2 (1×15 cm). The elution pattern of the pure dodecasodium phytate (IP_6) is shown in Figure 1(A). The separation of dodecasodium phytate as standard reference gave one beak that eluted in fraction No. 38 - 47. The chromatographic separation of a standards consisted of inositol-*1,4,5*-triphosphate (IP_3) and dodecasodium phytate (IP_6) is in Figure 1(B). IP_3 was eluted in the fractions No. 22-25 and IP_6 in the same identical position as in Figure (1A). To identify the positions of other forms of inositol phosphates, the partially hydrolyzed dodecasodium phytate was chromatographic separated on Dowex column (Fig. 1C) and followed by assay the molar ratio of inositol to phosphorus of each combined peak (Table 1). The chromatographic separations of hydrolysate (Fig. 1C) indicated the presence of six inositol phosphate forms (IP_1-IP_6) plus the liberated inorganic phosphate (P_i). The use of Dowex 1X2 as anion exchange column chromatography allows the separation of phytates into inositol mono- (IP_1), inositol di- (IP_2), inositol tri- (IP_3), inositol tetra- (IP_4), inositol penta- (IP_5) and inositol hexaphosphate (IP_6) [25]. Molar ratio assay of inositol to phosphorus in each separated peak (Table 1) is a good indication to identify the form of inositol phosphate. The same technique was implemented previous by Nayini and Markakis [29] and they found six types of inositol phosphates (IP_1-IP_6) during fermentation of bread.
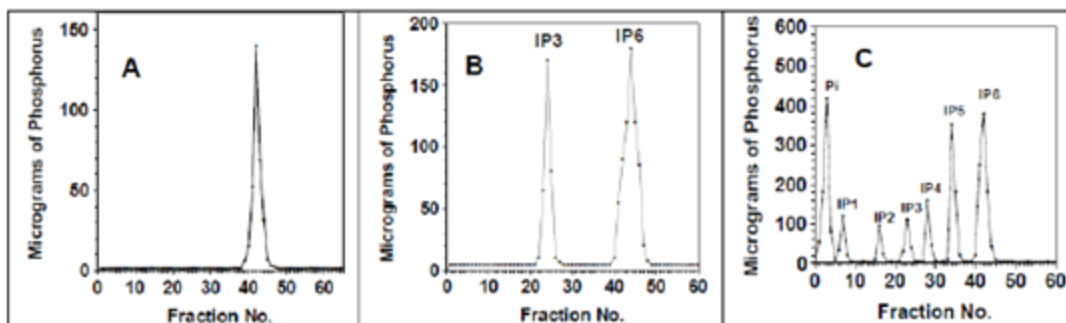
Figure 1: Chromatographic separation of different standards of inositol phosphates on a column of Dowex 1X2. A: dodecasodium phytate (IP$_6$), B: IP$_3$ and IP$_6$, C: Autoclaved dodecasodium phytate

Table 1: Molar ratio of inositol to phosphorus in intermediate of partial hydrolyzed dodecasodium phytate separated on Dowex 1X2.

Fraction No.	Ratio Inositol : Phosphorus
5-9	1 : 0.95
15-18	1 : 1.89
21-25	1 : 2.91
27-30	1 : 4.05
33-37	1 : 4.82
39-46	1 : 5.89

3.2 Effect of soaking and germination on the changes of inositol phosphate forms in legumes:
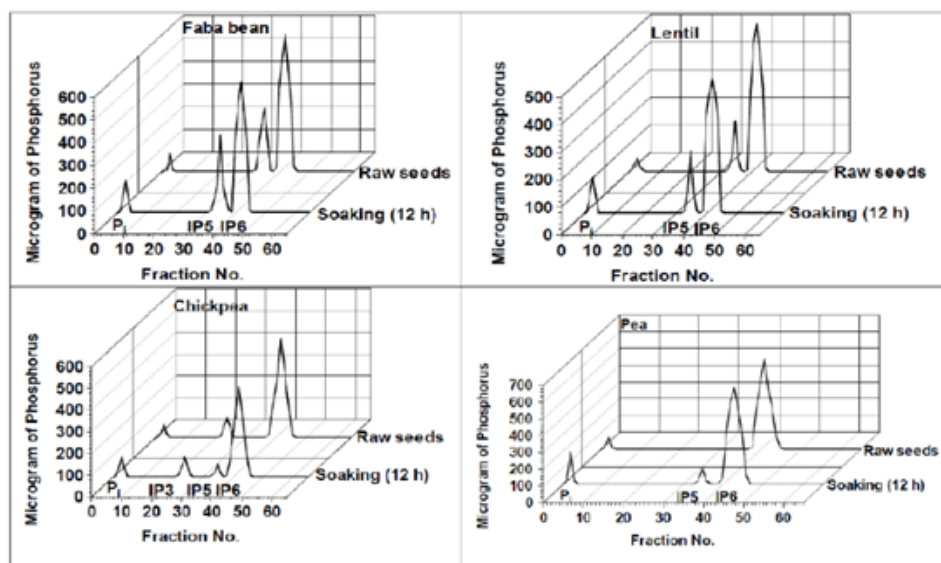

Figure 2: Effect of soaking on degradation of phytates in legumes

Phytate is a difficult molecule to analyze because it has no spectrum and may be hydrolyzed into lower esters with similar chemical properties, *i.e.*, IP$_5$, IP$_4$ and IP$_3$. Many factors may cause partial or complete hydrolysis, i.e., acids, bases and enzymes [30]. The separation of individual inositol phosphate form extracted from raw seeds of faba bean, lentil, chickpea and pea are shown in Figure (2). Faba bean and lentil contained two types of inositol polyphosphates, IP$_5$ and IP$_6$, whereas in raw chickpea IP$_3$ was found besides the IP$_6$. In raw pea seeds only IP$_6$ was

detected. Inorganic phosphate (P_i) was detected in all tested raw legume seeds. Burbano et al [31] determined the inositol phosphates in legumes by HPLC and found that IP_6 was the dominant inositol phosphates in lupine, lentil, beans, chickpea and faba bean. They found IP_5 and IP_6 in lupin; IP_3, IP_4, IP_5 and IP_6 in lentil; IP_3, IP_5 and IP_6 in beans; IP_3, IP_5 and IP_6 in chickpea and IP_3, IP_4, IP_5 and IP_6 in faba bean. The change in inositol phosphate forms by soaking of legume seeds for 12 hrs in tap water are given in Figure 2. The comparison of separation pattern of inositol phosphates of raw legume seeds with that of soaked seeds showed that IP_6 diminish whereas IP_5 and inorganic phosphate (P_i) increase in faba bean and lentil. In chickpea and pea, the decrease of IP_6 was accompanied by formation of IP_5 and increase of organic phosphate after 12-hrs soaking. These changes in separation pattern of inositol phosphates by soaking may be due to activation of enzyme phytase which degraded IP_6 into lower inositol phosphates and inorganic phosphate [12].

The separation pattern of inositol phosphates from 72-hrs germinating legumes showed a large degradation of IP_6 and increases in lower inositol phosphates and inorganic phosphate compared to 48-hrs germination (Fig. 3).
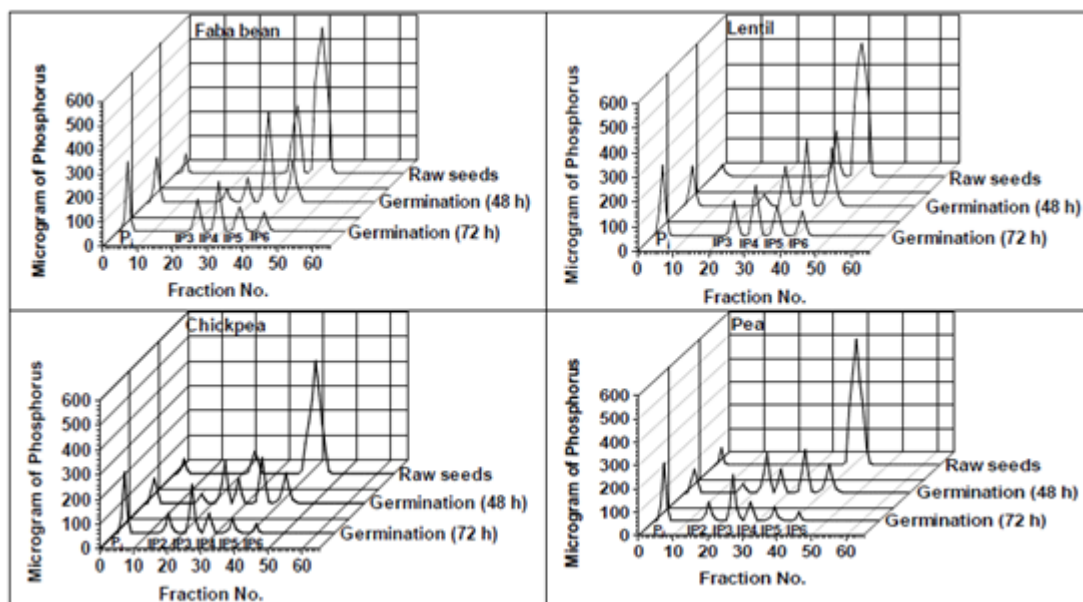

Figure 3: Effect of germination on degradation of phytates in legumes

3.3 Quantitative determination of inositol phosphate forms in raw, soaked and germinating legumes:

The contents of different inositol phosphates in raw legumes (Table 2) showed that the faba bean contained higher concentration of IP_6 (836.9) followed by Pea (825.2), lentil (814.8), and the least chickpea (637.0 mg/100g d.m.). IP_5 was found in faba bean and lentil with concentrations of 222.6 and 109.4 mg/100g d.m.; respectively. IP_3 was detected only in chickpea with concentration of 117.8 mg/100g d.m. In respect to the total inositol phosphates (TIP), faba bean had the highest content followed by lentil and pea whereas the chickpea had the lowest. Skoglund et al **[32]** used an anion-exchange resin AG 1X8 (200–400 mesh) for determination of inositol mono- to hexaphosphate (IP_1–IP_6) in selected foods and concluded that such method can be used to study the effect of food processing on formation of lower inositol phosphates in cereals and legumes. Morris and Hill [33] found IP_3, IP_4, IP_5 and IP_6 in raw seeds of chickpea, lentil and in different varieties of beans. They reported that the concentrations of these types of inositol phosphates were variable with high concentration of IP_6 followed by IP_5 in all sample tested. Frias et al [12] studied the inositol phosphates in lentil and peas. They found in lentil 269 mg IP_6, 75 mg IP_5, 48 mg IP_4 and 27 mg IP_3/100g d.m. Inositol phosphate contents in raw legume seeds reported in this work were within the ranges found in literatures [12, 31, 33].

Table 2: Inositol phosphates content of different raw, soaked and germinating legumes

Samples[a]	Inositol polyphosphates[b]				Total (TIPs)[d]
	IP3	IP4	IP5	IP6	
Faba bean:					
Raw	nd[c]	nd[c]	222.6 (2.4±)	836.9 (2.2±)	1059.5 (4.6±)
Soaked (12 hrs)	nd[c]	nd[c]	269.4 (2.3±)	759.1 (1.8±)	1028.5 (4.1±)
Germinating (48 hrs)	47.2 (1.3±)	75.70 (0.3±)	312.4 (2.2±)	151.8 (2.3±)	587.1 (1.5±)
Germinating (72 hrs)	117.8 (1.6±)	151.5 (1.5±)	85.90 (1.2±)	59.20 (3.3±)	414.4 (4.6±)
Lentil:					
Raw	nd[c]	nd[c]	109.4 (2.3±)	814.8 (1.6±)	924.2 (0.7±)
Soaked (12 hrs)	nd[c]	nd[c]	148.4 (2.8±)	729.5 (2.6±)	877.9 (5.4±)
Germinating (48 hrs)	51.8 (3.2±)	134.6 (3.4±)	179.5 (1.5±)	188.8 (1.8±)	554.7 (3.1±)
Germinating (72 hrs)	122.5 (2.4±)	168.3 (3.3±)	93.7 (3.0±)	74.1 (2.9±)	458.6 (1.0±)
Chickpea:					
Raw	117.8 (2.6±)	nd[c]	nd[c]	637.0 (3.6±)	754.8 (6.2±)
Soaked (12 hrs)	112.5 (2.5±)	nd[c]	39.0 (2.8±)	555.6 (4.3±)	707.1 (4.6±)
Germinating (48 hrs)	150.7 (0.4±)	75.7 (3.3±)	132.8 (2.7±)	103.7 (4.2±)	462.9 (5.2±)
Germinating (72 hrs)	169.6 (2.3±)	67.3 (3.1±)	39.1 (3.3±)	14.8 (0.9±)	290.8 (3.4±)
Pea:					
Raw	nd[c]	nd[c]	nd*	825.2 (2.6±)	825.2 (2.6±)
Soaked (12 hrs)	nd[c]	nd[c]	47.5 (2.1±)	698.0 (2.7±)	745.5 (4.8±)
Germinating (48 hrs)	nd[c]	175.8 (3.6±)	174.5 (2.7±)	110.0 (3.1±)	460.3 (2.2±)
Germinating (72 hrs)	90.4 (1.3±)	97.3 (2.3±)	94.6 (0.7±)	20.4 (1.9±)	302.7 (2.2±)

[a]Two extracts of each sample and analyzed in duplicate.

[b]Mean values expressed as mg per 100 g sample on dry weight basis with their standard deviations (SD).

[c]Not detected.

[d]Total inositol phosphates (TIPs)

Soaking decreased the total inositol phosphates by 2.9% in faba bean, 5.0% in lentil, 6.3% in chickpea and 9.6% in pea of the original amount (Table 2). Furthermore, the results indicated that an increase in IP_5 of soaked faba bean and lentil amounting to 21 and 35.6% of the original content; respectively. IP_5 was formed in soaked chickpea (39.0) and pea (47.5 mg/100g d. m.), as a result for IP_6 degradation during soaking process. A slight decrease was found in content of IP_3 of soaked chickpea compared to that of raw seeds. Pedrose et al [34] reported increase of the amounts of IP_4 and IP_5 after processing of lupine, soybean, lentil, chickpea and roasted peanut due to dephosphorylation of IP_6. Soaking enhance the action of naturally occurring phytase in legumes. It has been shown that phytate hydrolysis, during soaking, is greatly influenced by temperature and pH [35]. The results of the current study are in agreement with the transformation of phytic acid reported by other investigators [18, 36]. Although the soaking decreased the total phytate content by leaching out in soaking water, but that remained inositol phosphate in seeds during soaking may be attacked by activated endogenous phytases. These results are in agreement with the fact that phytic acid (IP_6) decrease and low inositol phosphates as well as inorganic phosphate increase during the first stage of IP_6 degradation. The increase in inorganic phosphorus may be explained by the formation of orthophosphate from hydrolysis of phytic acid by phytase activity [34]. Kalpanadevi and Mohan [14] reported that soaking induced reduction in phytate content in legumes that attributed to the activity of phytase and diffusion of the products. In contrast, Vidal-Valverde et al [37] reported that soaking and soaking plus cooking did not significantly change the phytic acid level in faba beans.

The removing of total inositol phosphates by germination for 48 and 72 hrs accounted to 44.6 ، 60.9% for faba bean, 40 ، 50.4% for lentil, 38.7 ، 61.5% for chickpea and 44.2 ، 63.3% for pea; respectively (Table 2). The contents of IP_6 decreased and IP_5 increased with formation of IP_3 (47.2 in faba bean and 51.8 in lentil) and IP_4 (75.7 in faba bean and 134.6 mg/100 mg d.m. in lentil) after 48-hrs germination. In addition, germination for 48 hrs caused lowering in contents of IP_6 and IP_5 in chickpea with formation of IP_4 with concentration of 75.7 mg/100 g d.m., and an increase in the content of IP_3. The same trend was observed in pea for IP_6 and IP_5 after 48 hrs germination. IP_4 was detected after 48-hrs germination in pea and accounted to 175.8 mg/100g d.m. IP_3 was not found in pea after this period of germination.

Sharp decreases in IP_6 and IP_5 and increases in IP_4 and IP_3 were estimated for faba bean and lentil after germination for 72 hrs (Table 2). 72-hrs-germinating chickpea and pea showed also a large dropping in the contents of IP_6 and IP_5, comparing to 48-hrs germination, with decrease in the content of IP_4 and increase in IP_3. The dephosphorylation of phytate is a prerequisite for improving nutritional value because removal of phosphate groups from the inositol ring decreases the mineral binding strength of phytate. This results increased bioavailability of essential dietary minerals [38]. Germination appears to be an effective method of achieving desirable changes in the nutritive value of legume seeds. A number of studies have indicated that the content of inositol phosphates changes in a range of legumes and the extent of these changes is mainly determined by the conditions of germination [39]. Harland and Narula [40] reported that the processing of food leads to hydrolysis of phytate to lower inositol phosphates. They found in processed legumes such as cooked lentil, peas, and kidney beans variable amounts of inositol phosphates (IP_3, IP_4, IP_5 and IP_6). Evidence suggests that at least some of these hydrolysis products have much less affinity or less potential for reducing the bioavailability of minerals [41]. Furthermore, lower inositol phosphates, such as IP_4 and IP_3, may play roles in mediating cellular responses and have been noted to have a function in second messenger transduction systems [42]. Ishizuka et al [43] reported that mainly inositol triphosphate (IP_3) with three phosphates on positions 1,2,6 and 2,5,6 are responsible for inhibition the proliferation of colorectal cancer cells.

4. Conclusion

Soaking of legumes for 12 hrs followed by germination for 72 hrs lowered the content of IP_6 and IP_5 (inositol phosphates causing impaired absorption of essential minerals) and increased the IP_4 and IP_3 (inositol phosphates which are not involved in the mineral bioavailability and which may play roles in mediating cellular responses as second messengers). In order to obtain legume or legume flour with a low content of IP_6 and IP_5 and high content of IP_4 and IP_3, the described processing should suffice for this purpose.

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