PURIFICATION OF PHYTASE PRODUCED FROM A LOCAL FUNGAL ISOLATE AND ITS APPLICATIONS IN FOOD SYSTEMS

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ABSTRACT

Phytase produced from local isolate of *Aspergillus tubingnesis SKA* was purified to homogeneity by three steps including 80% ammonium sulphate precipitation, followed by ion exchange chromatography by using DEAE-Cellulose and gel filtration with a Sephacryl S-200, the purified enzyme was obtained with 9.18 fold of purification and a yield of 25.92%. The purified phytase showed an optimal pH and temperature for its activity with about 5.0 and 40 °C respectively. The purified phytase was used to produce high quality (low phytate) soy protein concentrate (SPC) and soy protein isolate (SPI) from defatted soy flour (DSF). Enzymatic treatment increased the protein content and the yield from 52.00% and 40.32% to 55.00% and 46.58% in the untreated and treated SPC respectively, meanwhile for SPI the protein content and the yield increased from 72.00% and 50.00% to 87.00% and 63.91% respectively. On the other hand a significant decrease were seen in phytate content from 0.94% (untreated) to 0.42% (treated) SPC, and for SPI phytate content decreased from 1.10% to 0.39% respectively. Purified phytase enzyme was successfully capable to fractionate soybean globulins (glycinin and β-conglycinin), as phytate content decreased from 1.30% in defatted soymilk (DSM) to 0.33% and 0.37% in soymilk treated with *A. tubingensis SKA* phytase and wheat phytase respectively, protein content of soy globulins fractions (glycinin and β-conglycinin) was increased to (14.27 and 12.05%), (13.44 and 11.80%) respectively. Comparing to the treatment of buffer and reducing agent, protein content was (12.57 and 10.39%) and no change was observed on phytate content by this method.

Key words: Phytic acid, *Aspergillus tubingensis SKA*, (glycinin and β-conglycinin) and soy protein isolate.
***Part of Ph.D. Thesis of the first author.**

تنقية الفايتيز المنتج من عزلة فطر محلية وتطبيقاته في الأنظمة الغذائية

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المستخلص

تم تنقية الفايتيز المنتج من عزلة محلية للفطر *Aspergillus tubingnesis SKA* الى حد التجانس بثلاث خطوات تضمنت الترسيب بـ 80% من كبريتات الامونيوم تلتها خطوة التنقية باستخدام المبادل الايوني DEAE–Cellulose وأخيرا الترشيح الهلامي باستخدام هلام Sephacryl S–200. وكان عدد مرات التنقية والحصيلة الانزيمية 9.18 و25.92% على التوالي بينما كان الاس الهيدروجيني الأمثل ودرجة الحرارة المثلى لفعالية الفايتيز المنقى 5.0 و40 °م على التوالي. استعمل الانزيم المنقى في انتاج مركزات ومعزولات بروتينية عالية الجودة (ذات محتوى واطئ من حامض الفايتيك) من فول الصويا، وقد أدت هذه التجارب الى ارتفاع في محتوى البروتين والحصيلة من 52.00% و40.32% (غير المعامل انزيمياً) الى 55.00% و46.58% (المعامل انزيمياً) على التوالي في مركزات البروتين، في حين كانت الزيادة في محتوى البروتين والحصيلة في معزولات البروتين من 72.00% و50.00% (غير المعامل انزيمياً) الى 87.00% و63.91% (المعامل انزيمياً) على التوالي في النماذج. من جانب اخر كان هنالك انخفاض واضح في محتوى هذه المنتجات من حامض الفايتيك من 0.94% الى 0.42% في مركزات البروتين المعاملة وغير المعاملة انزيمياً، في حين كان الانخفاض من 1.10% الى 0.39% في مركزات البروتين المعاملة وغير المعاملة انزيمياً على التوالي. كما اظهر انزيم الفايتيز النقي قدرته على فصل كلوبيولينات فول الصويا (glycinin وβ–conglycinin) بنجاح، ففي الوقت الذي انخفض فيه محتوى حامض الفايتيك من 1.3٪ في حليب الصويا منزوع الدهن الى 0.33٪ و0.37٪ المعامل بفايتيز العزلة الفطرية *A. tubingensis SKA* وفايتيزالقمح على التوالي، فان محتوى البروتين في الأجزاء المفصولة من كلوبيولينات الصويا (glycinin وβ-conglycinin)، ازداد الى (14.27 و12.05٪)، (13.44 و11.80٪) على التتابع. مقارنة بالفصل باستعمال المحلول الدارئ والعامل المؤكسد، حيث كان محتوى البروتين (12.57و 10.39٪) ولم يكن هنالك أي تغير ملحوظ على محتوى حامض الفايتك بواسطة هذه الطريقة.

الكلمات المفتاحية: حامض الفايتيك، *Aspergillus tubingensis SKA*، (glycinin and β-conglycinin) ومعزول بروتين فول الصويا.
***البحث مستل من أطروحة دكتوراه للباحث الأول.**

INTRODUCTION

Phytases are sub-group of phosphatases that have the capability to hydrolyze the monophosphoester bonds of phytic acid (*myo*-inositol hexakisphosphate IP6) to lower inositol polyphosphates or in some cases free inositol and release inorganic phosphate (Pi) (3, 12). Phytic acid (*myo*-inositol hexakisphosphate (IP6), *myo*-inositol polyphosphate), is the principle storage form of phosphorus in most of plant tissues, and has been found to be a nearly ubiquitous component in cereals, legumes and oilseeds crops, constituting 1-5% of their weight and representing about 60-90% of the total phosphorus in plant tissues (10, 22). Therefor phytic acid is a common constituent of plant-derived foods, which represents the staple foods especially in developing countries (16). Due its chemical structure, phytic acid interacts with other food ingredients, which makes it an anti-nutritional factor (ANF) in different ways (19). Phytic acid has the ability to interact with proteins to form complexes with over a wide range of pH. In general, pH is a crucial factor for protein and phytate interactions, such interactions with dietary protein reduce their solubility and digestibility as well as their nutritive values, and would alter protein structure, thus hampering the activity of endogenous proteases because of the steric hindrance (15, 23). In addition phytic acid has tremendous affinity to form quite stable complexes with positively charged food components such as trace elements and metal ions (Ca^{+2}, Fe^{+2}, Zn^{+2}, Mg^{+2}, Mn^{+2}, Ni^{+2}, etc.). The major concern of the presence of such complexes in human diet is the negative effect on mineral uptake, which would be the main reason for minerals deficiencies for people who rely in their diet on plant-derived foods (4, 5). The presence of phytic acid in plant derived food such as the derived products from soybean which have been the chief source of protein to many countries especially Eastern Asia, can cause a real problem, thus enzymatic treatment with phytases was considered the superior method to solve such problem (13, 16). The products of soybean proteins have become so popular, because of their nutritional value, functional properties and low prices. The most important products are soy protein concentrate (SPC) and soy protein isolate (SPI), these two products have been increasingly used in (infant's formulas, meat and dairy products) as nutritional additives, emulsifiers, stabilizers, water and fat absorbents, etc. (2, 9, 17). Soybean proteins have an important therapeutically role, they have been found that soybean proteins decrease the plasma cholesterol liver (13). The two major proteins of soybean are (glycinin and β-conglycinin), fractionation of these proteins to prepare pure fractions to study their biochemical and nutritional characteristics have been conducted by using reducing agent and cooling into the soluble and insoluble fractions. Using phytases for soy protein fractionation or for the production of SPC and SPI was a new approach to produce high quality products (10, 24). The objectives of this study were the purification and partial characterization of phytase produced from *Aspergillus tubingensis SKA*, then utilization of the obtained enzyme in the production of high quality (low phytate) of SPC, SPI and fractionation of soybean globulins.

MATERIALS AND METHODS

Instruments and chemical

Cooled centrifuge (Thermo Fisher Scientific), hot plate stirrer (Cimaric), Electrophoresis (Bio-Rad), Lyophilizer (Christ Alpha), pH meter (Sartorius), water bath and spectrophotometer (Thermo Fisher Scientific). Commercial defatted soy flour was purchased from (My Spice Sage, USA). Enzymes: wheat phytase was purchase from (Sigma –Aldrich, USA), fungal phytase was produced from Iraqi local isolate of *Aspergillus tubingensis SKA* under solid state fermentation, all other chemicals were purchased from Sigma-Aldrich, Fisher Scientific and Bio-Rad (USA).

Phytase assay

Phytase activity was estimated according to the modified method of Awad *et al*., (1) and the liberated inorganic phosphate was determined according to the method of Fiske and Subbarow, (7). One unit of phytase is defined as the amount of the enzyme that releases one µg inorganic phosphate per ml per min under the assay conditions.

Protein determination

Protein was estimated according to the method of Lowry *et al*., (18) using BSA as the standard.

Purification of phytase enzyme from fungal isolate

Aspergillus tubingnesis SKA

All purification steps were carried out at 4 °C. The crude enzyme was subjected to ammonium sulphate precipitation 80% the required amount of dry $(NH_4)_2SO_4$ salt was added slowly to the crude extract under mild stirring conditions and the stirring was continued for 30 min at 4°C. Precipitated protein was collected by centrifugation at 10,000xg for 15 min on a cooling centrifuge, then the supernatant discarded and an appropriate amount of (0.02 M, pH 7.2) Tris-HCl buffer was used to resuspend the precipitant. The resuspended protein was desalted using dialysis bag against (0.02 M, pH 7.2) Tris-HCl buffer for 24 h, and then loaded onto (1.5x30 cm) DEAE-Cellulose column with a flow rate of flow rate of 30 ml/h. Proteins were eluted using the gradient of 0.1 to 1.0 M NaCl in (0.02 M, pH 7.2) Tris-HCl buffer, the fractions that showed activity for phytase were pooled and concentrated by using poly ethylene glycol PEG 20,000 and the concentrate was loaded onto a (1.5x50 cm) Sephacryl S-200 pre-equilibrated with (0.2 M, pH 5.5) acetate buffer with a flow rate of flow rate of 30 ml/h. Proteins were eluted with the same buffer, the fractions with phytases activity were collected and used for further experiments.

Determination of the optimal ph and temperature for activity of the purified phytase

Purified enzyme was incubated for 30 min at 35°C, with pH ranging (2.0-10.0) buffer solutions, immediately after incubation they were cooled in an ice bath and then enzyme assay was performed according to Awad *et al*., (1) to estimate the optimal pH for the purified phytase activity. For the determination of optimum temperature for enzyme activity, phytase assay was conducted as under a range of temperatures (20-25-30-35-40-45-50-55-60) °C.

Production of high quality (low phytate) spc and spi

SPC and SPI were prepared according to the method of Wang *et al*., (30) with a modification of using phytase treatment, defatted soy flour (DSF) was dispersed in distilled water in the ration of 1:10 (soy/water), the slurry was adjusted to pH 5.5 with 2M HCl, phytase was added to this slurry (10 unit/gm soy protein), the resulting mixture was incubated for 1 h at 40 °C, then the procedure was continued as in (Fig 1-2).

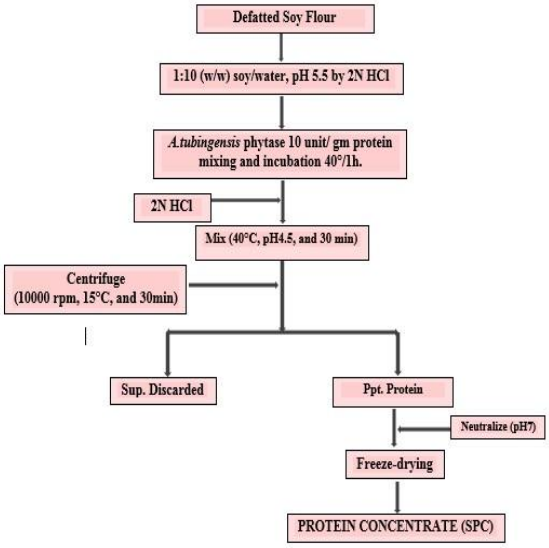

Figure 1. Schematic Diagram for the production of (SPC) according to Wang *et al*., (30).

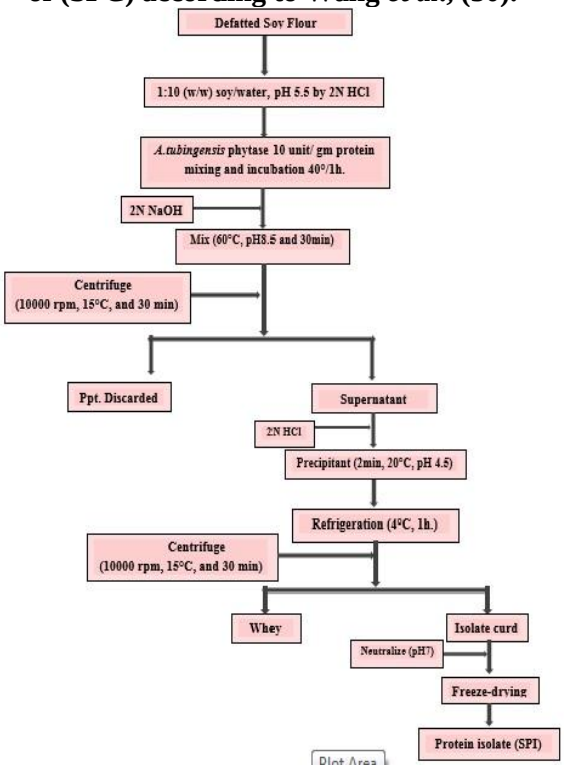

Figure 2. Schematic Diagram for the production of (SPI) according to Wang *et al*., (30)

Fractionation of soybean glob-ulins by phytase treatment

The separation of glycinin and β-conglycinin from defatted soy flour (DSF) by phytase treatment conducted according to the method of Saito *et al*., (24) as in Fig. 3, while the preparation of glycinin and β-conglycinin by using buffer and reducing agent was conducted according to the method of Thanh and Shibasaki, (28) as in Fig. 4.

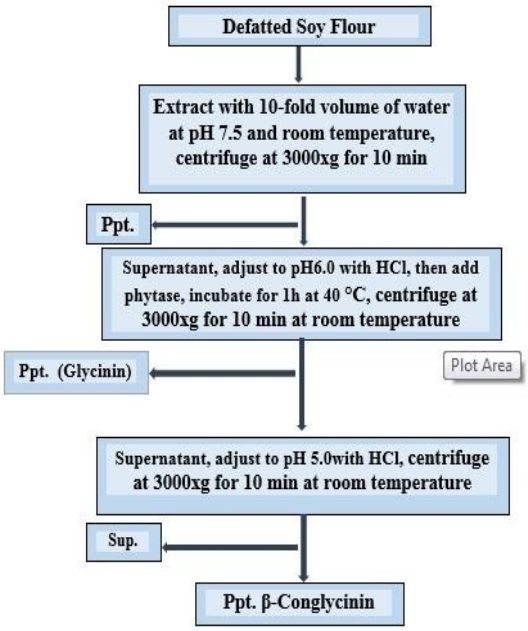

Figure 3. Schematic Diagram for the separation of soy globulin according to Saito *et al*., (24).

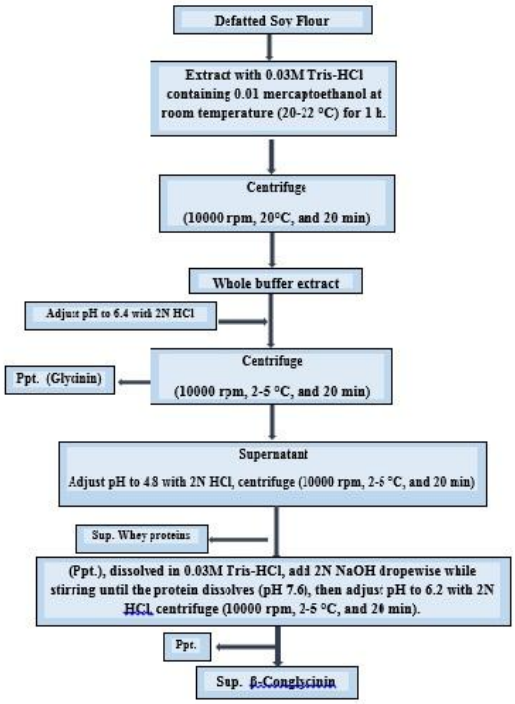

Figure 4. Schematic Diagram for the separation of soy globulin according to Thanh and Shibasaki, (28).

Phytate content was determined according to the method of Mohamed *et al*., (19). The protein determination: the total protein content was determined by Kjeldhal method, bicinchoninic acid BCA protein assay kit was used to determined extractable soluble protein content .The protein extractability was expressed as gm of extractable protein per 100 gm dry mass. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) provided by (*Bio-Rad*), the separating gel gradient was between 12% acrylamide gel.

RESULTS AND DISCUSSION

Aspergillus tubingnesis SKA phytase enzyme was purified in three steps as summarized in Table (1). In the first stage crude enzyme was precipitated with 80% ammonium sulphate, which was found in a previous experiment as the maximum ratio of saturation with a yield of 71.99% and 2.23 fold of purification. Then it was dialyzed against (0.02 M, pH 7.2) Tris-HCl buffer for 24 h, then concentrated and applied to DEAE-Cellulose column, the second step of purification.

Table 1. Purification steps of phytase from local isolate of *Aspergillus tubingnesis SKA*.

Step of purification	Volume (ml)	Protein (mg/ml)	Activity (unit/ml)	Specific activity (unit/mg)	Total activity (unit)	Fold of purification	Yield (%)
Crude	40	0.989	28.94	29.26	1157.6	1	100
$(NH_4)SO_4$ 80%	15	0.851	55.56	65.28	833.4	2.23	71.99
Ion exchange	14	0.215	35.65	165.81	499.1	5.66	43.11
Gel filtration	12	0.093	25.00	268.81	300.0	9.18	25.92

A considerable amount of protein was run out after washing with (0.02 M, pH 7.2) Tris-HCl buffer, protein was monitored at 280 nm and analyzed for phytase activity, but there was not any. Elution with a gradient of NaCl (0.1-1.0 M) in the same buffer resulted into two peaks of proteins. Major activity was in the second one (Fig. 5), in this step, phytase was purified with a yield of 43.11% and a fold of purification of 5.66, specific activity increased to 165.81 unit/mg compared with the crude enzyme 29.26 unit/mg.

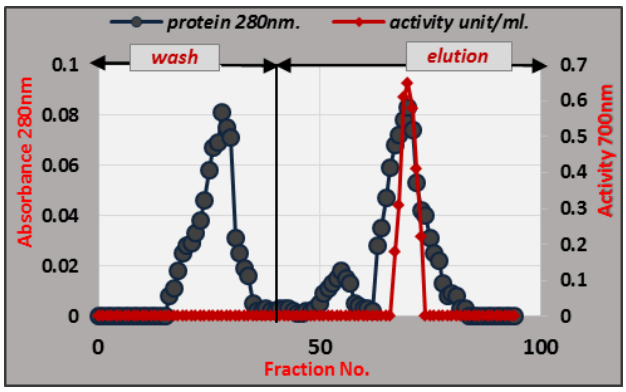

Figure 5. Purification of phytase by ion exchange chromatography with (1.5x30 cm) column of DEAE-Cellulose, flow rate 30 ml/h, 2 ml per fraction with gradient of NaCl (0.1-1.0 M) in (0.02 M, pH 7.2) Tris-HCl buffer.

Fractions showed activity were pooled, dialyzed, concentrated, and loaded into Sephacryl S-200 the third step of purification, proteins were eluted with (0.2 M, pH 5.5) acetate buffer, elution resulted in three peaks of protein. Major peak showed phytase activity (Fig. 6), in this step phytase was purified with a yield of 25.92% and a fold of purification of 9.18, specific activity increased to 268.81 unit/mg compared with the one of the crude enzyme

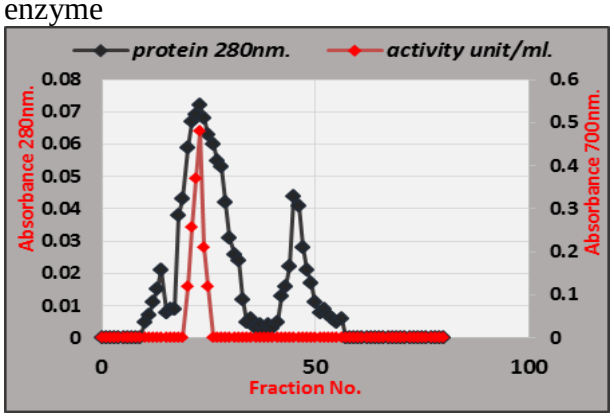

Figure 6. Purification of phytase by Gel filtration with (1.5x50 cm) column of Sephacryl S-200, flow rate 30 ml/h, 2 ml per fraction with (0.2 M, pH 5.5) acetate buffer

Phytase enzyme was purified either partially or to homogeneity by different techniques. Sariyska *et al*., (25) purified *Apergillus niger* phytase to homogeneity by three steps of purification ; ultrafiltration, gel filtration with Sephadex G-100 and ion exchange chromatography with DEAE-Sepharose CL 6B, with a yield of 3.81% and a fold of purification of 30. Zhu *et al*., (23) conducted a full purification for the phytase produced from the fungus *Flammulina velutipes* to homogeneity by five steps of purification with a yield of 19% and 30.9 fold of purification. Other related study reported two-steps of purification with DEAE-Sepharose FF and Sephacryl S-100 HR to purify phytase produced from *Bacillus subtilis* MJA with a yield of 57.7% and 3.95 fold of purification, (26). Onem and Nadaroglu, (20), were able to purify phytase produced from the fungus *Lactarius quientus* to homogeneity by two steps: ammonium suplate precipitation and Ion exchange chromatography, with a yield of 31.1% and a fold of purification of 32.54. The purified phytase from *Aspergillus tubingnesis SKA* displayed optimum activity at pH 5.0 with a value reached (52.16 unit/ml), while a sharp decrease was observed in the activity (8.67 and 5.34 unit/ml) at extreme pH 2.0 and 8.0 respectively (Fig. 7). This observation is similar to the findings of many related studies in this area.

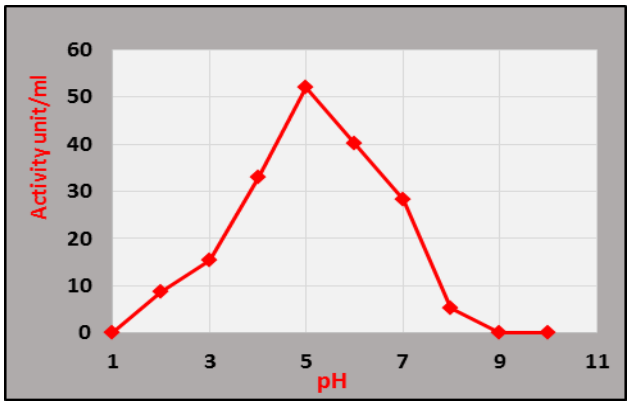

Figure 7. The optimal pH for purified phytase activity.

Vohra and Satyanarayana, (29) reported in their review that phytase produced by *Aspergillus niger* NRRL 3135 had two optimal pHs for activity (2.2 and 5.0-5.5), while *Aspergillus niger* 92 phytase displayed an optimal pH for activity at (5.0). The same review showed that optimum activity of phytase produced by (*Aspergillus carneus*, *Aspergillus carbonicus*, *Aspergillus terreus* and *Pencillium sp.*), was at pH (5.6, 4.7, 4.5 and 3.0) respectively. Our findings were in consistent with other related studies who confirmed that phytase activity has reached its maximal values at pH 5.0, (27, 32). The purified phytase optimum activity (44.3 unit/ml) at 40 °C. Adversely, enzymatic activity was decreased over and below this

temperature, while enzymatic activity was totally lost at temperatures over 60 °C (Fig. 8).

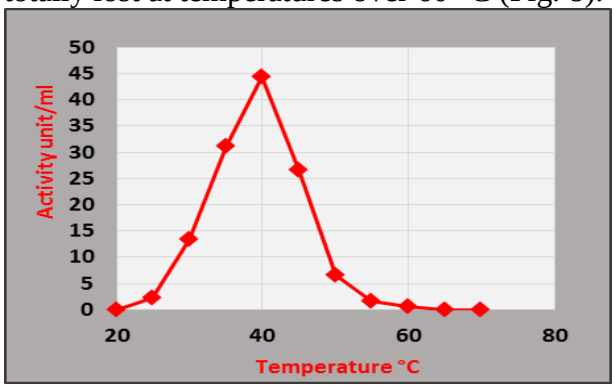

Figure 8. The optimal temperature for purified phytase activity.

These findings agreed with Vohra and Satyanarayana, (29) who reported that the optimum temperature for activity of phytase produced by *Aspergillus carneus* and *Pseudomonas sp.* was 40 °C. Almost similar findings were reported by other related studies. Rani and Ghosh, (21) observed that optimum temperature for activity of *Rizopus oryzae* phytase was at 45 °C. This result was confirmed by Zhu *et al*., (32) who demonstrated that optimum temperature for activity of phytase produced by winter mushroom *Flammulina velutipes* was at 45 °C. While Shah and Trivedi, (26) found that *Aspergillus tamari* phytase had an optimum temperature for its activity at 28 °C. Phytase of *Aspergillus tubingnesis SKA* was used to produce high quality (low phytate) soy protein concentrate (SPC) and soy protein isolate (SPI) as shown in Fig. (9), which have a high content of protein and low content of phytate according to the method of Wang *et al*., (30), with a modification of using enzymatic treatment. It was obviously shown that enzymatic treatment has increased the protein content and yield from 52.00% and 40.32% to 55.00% and 46.58% in phytase untreated and treated SPC respectively. The same increase was achieved in protein content and yield, from 72.00% and 50.00% to 87.00% and yield 63.91% in phytase untreated and treated SPI. On the other hand, there was a significant decrease in phytate content from 0.94% to 0.42% in the untreated and treated SPC, and for the untreated and treated SPI the decrease was from 1.1% to 0.39% respectively (Table 2).

Figure 9. Treated and untreated (SPC) and (SPI) with phytase of *A. tubingnesis SKA*.

Table 2. Protein and phytate content of treated and untreated products with *A.tubingensis SKA* phytase.

Product	N%	Protein%	Yield%	Phytate%
DSF	7.76	49.00	100	1.40
SPC	8.29	52.00	40.32	0.94
SPI	11.47	72.00	50.00	1.10
SPC*	8.81	55.00	46.58	0.42
SPI*	13.89	87.00	63.91	0.39

***Phytase treated products, Defatted soy flour (DSF), Soy protein isolate (SPI), Soy protein concentrate (SPC).**

Results also illustrated in Fig (10), demonstrated the increase in protein content that was associated with the decrease of phytate content after enzymatic treatment. Phytase has the ability to form complexes with proteins and reduce their solubility and digestibility; such complexes formations may affect the protein structures that can hamper the digestive enzymes such as pepsin, trypsin, and chymotrypsin, (14, 16). Phytase treatment

seemed to have significant advantages of releasing and increasing the extractability of protein bound to cellular components, minerals and phytate. Therefore, released proteins can be more soluble in SPC and SPI that increase the quality of these products, such as the nutritional value of the products that contain such concentrates and isolates. These results were similar to the results of Hurrell *et al*., (11) whose findings showed an increase in protein content from 90.10% to 91.80% in untreated and treated SPI with *Aspergillus niger* phytase, and protein content increased from 87.70% to 92.60% in native phytate and low-phytate SPI produced by continuous acid-salt and ultrafiltration. Wang *et al*., (31), found that removing phytate from defatted rice bran by using phytase produced by *Aspergillus niger* increased the protein content and yield from 74.50% and 34.20% to 80.20% and 57.30% respectively. Gandhi *et al*., (8) achieved an increase in the protein content from 57.40% to 58.00% when phytate decreased from 1.20% to 0.20% respectively. Fractionation of soybaen globulins was carried out by two methods according to Saito *et al*., (24) and Thanh and Shibasaki, (28). Results in Table (3) showed the content of phytate and protein of soy globulins fractions. Using phytases have beneficial aspects; beside the simplicity of this method, reducing phytate content had a shiny reflection on the results.

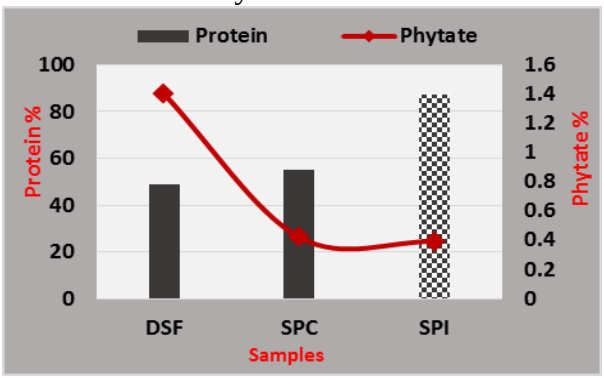

Figure 10. Effect of *A. tubingensis SKA* phytase treatment on the content of phytate and protein of SPC and SPI.

As phytate content decreased from 1.3% in defatted soymilk (DSM) to 0.33% and 0.37% in soymilk treated with *Aspergillus tubingnesis SKA* phytase and wheat phytase respectively, protein content of soy globulins fractions (glycinin and β-conglycinin) was increased to (14.27 and 12.05%), (13.44 and 11.80%) respectively. Comparing to the treatment of buffer and reducing agent, protein content was (12.57 and 10.39%) and no change was observed on phytate content by this method. The increase in protein content is attributed to breaking down the protein-phytate complex, which increased the protein solubility. Saito *et al*,. (24) were able to decrease phytate content from 2.05% to 0.05% in defatted soymilk after treatment with wheat phytase and they were able to gain 16% and 11% protein of glycinin and β-conglycinin respectively. Protein contains both polar and nonpolar amino acids; one of the ways that proteins minimize their energy is by folding into structures of low energy. These structures generally resulted when protein is found in association with other compounds such as phytate, hindrance to folding can occur, that is why when these proteins released from phytate became more soluble (31). Phytate forms complexes with glycinin such complexe change the hydration and net charge of glycinin and affect its solubility, that's why glycinin cannot be precipitated at pH 6.0 on room temperature. Phytate removal by enzymatic treatment would break down glycinin-phytate complex and enable the glycinin to recover its net charge with increasing its extractability, so it can be precipitated at pH (6.0). β-conglycinin fraction would be prevented from such precipitation, and that refer to its isoelectric point which is lower than pH 6.0, beside that the oligosaccharide moiety of β-Conglycinin which is a glycoprotein would prevent its precipitation on pH 6.0 (15, 24). The simplicity of enzymatic treatment with phytases makes it superior compared to the conventional method of Thanh and Shibasaki, (28) which was widely used for the fractionation of soybean globulins. This method is based on precipitation of glycinin while β-conglycinin remain soluble, cooling and reducing agent are required to avoid cross contamination of glycinin and β-conglycinin during separation process. It is well known that soybean globulins are prone to form disulfide-linked polymers, for that reducing agent is needed for the depolymerization, but it is necessary to remove it to prevent the negative its effect on gel-foaming properties of the protein. Moreover the cooling ties up the

practicality of this procedure especially for industrial scale preparation, while using phytases in the separation of soybean globulins is more suitable to use for large-scale production with no need for cooling or reducing agent, (24).SDS-PAGE profiles of the glycinin and β-conglycinin fractions are shown in Fig. (11).The two methods were capable of fractionating soy globulins. The subunits of β-conglycinin and glycinin were clearly shown especially by using enzymatic treatment with isolate *A. tubingensis SKA* phytase. The beneficial aspects of this method (low phytate content of the products) and its simplicity would make it superior compared to the treatment of reducing agent for the separation of soybean protein fractions.

Table 3. Protein and phytate content of soybean globulins fractions.

Treatment	Glycinin %	β-Conglycinin %	Phytate %
A. tubingensis SKA phytase	14.27	12.05	0.33
Wheat phytase	13.44	11.80	0.37
Buffer&Reducing agent	12.57	10.39	1.1

*** Phytate content of the defatted soymilk (DSM) 1.3%.**

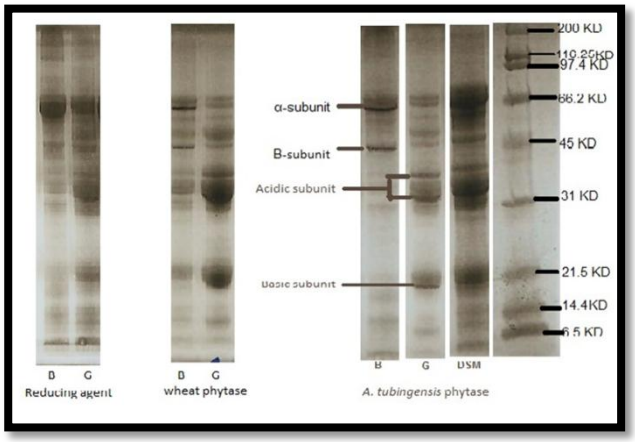

Figure 11. SDS-PAGE of the soy globulins fractions, lane B, β-Conglycinin; lane G, Glycinin.

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