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Preparation and Properties of Purified Phytase from Oakbug Milkcap (*Lactarius Quietus*) Immobilised on Coated Chitosan with Iron Nano Particles and Investigation of Its Usability in Food Industry

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Abstract In this study, phytase enzyme was purified and characterized from Oakbug Milkcap mushrooms (*Lactarius quietus*) and the purified phytase enzyme was immobilized on the surface of modified chitosan with nano-Fe_3O_4 nanoparticles. The phytase was purified from Oakbug Milkcap mushrooms using ammonium sulphate precipitation in the range of 40-80%. and DEAE-sephadex ion-exchange chromatography. The purification fold was calculated by determining the activity and amount of protein for each step. The purified phytase enzyme was then immobilized on modified chitosan support material with Fe_3O_4 nanoparticles. The optimum pHs for the immobilized and free enzyme were 6.0 and the optimum temperatures were 60°C. The effects of some metal ions such as $CuCl_2$, Hg_2Cl_2, $FeCl_2$, $MgCl_2$, $ZnCl_2$, $CaCl_2$ were investigated on both the bound and free purified phytase. The molecular weight of the enzyme was determined using the SDS-PAGE electrophoresis method. Finally, we investigated whether or not the immobilized and free enzyme could hydrolyzed the phytic acid in green lentils, red lentils, peas, pinto beans, beans, brass, corn, dried corn, oat, rye, wheat, broad bean, chickpeas and peanuts. It was concluded that the immobilized phytase was quite resistant to temperature, pH and metal ions and it could be safely used in the hydrolysis of phytic acid in the legumes in the food industry.

Keywords: *Oakbug Milkcap (Lactarius quietus) mushroom, phytase, phytic acid, immobilization*

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1. Introduction

Phytase (myo-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.8) is an enzyme which catalyzes the hydrolysis reaction of phytic acid (myo-inositol hexaphosphate) to inorganic monophosphate, myo-inositol phosphate and free myo-inositol. Phytic acid locates as main storage the source of phosphorus in food material including grains, legumes and oil seeds.

Phytate creates a negative effect on the mineral intake from food. In the blood pH, phytase caused mineral deficiencies in metabolism by causing an insoluble mineral-phytate complex formation with zinc, iron, calcium, magnesium, manganese and copper metal ions. These formations act as both enzyme cofactors and as different metabolic processes. Phytates also completely prevent the formation of phytate-protein complexes of protein and amino acids [1,2].

The phytase enzyme is located in animals, plants, microorganisms and fungi. Phytase was first identified in rice bran in the early twentieh century. It was later identified and determined in animals, plants and microorganisms including bacteria, yeast and fungi. Phytase was purified from many microorganisms including especially *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus oryzae*, *Escherichia coli*, *Emericella nidulans* and *Thermomyces lanuginosus* [3]. The first commercial phytase enzyme was obtained from *Aspergillus niger* and it was put up for sale in 1991. Phytase enzymes produced from several yeast species such as *Saccharomyces cerevisiae*, *Candida tropicalis*,*Kluyveromyces fragilis*, *Torulopsis candida*, *Debaryomyces castelli* and *Schwanniomyces castelli* were isolated and characterized [4]. In general, humans and animals have endogenous phytase activity and it is less important than plant, yeast, fungi, microbial phytase activity [5].

Phytase was purified and characterized from a few types of mushrooms. These mushrooms were edible and cultured, and included *Agrocybe pediades*, *Cenporia sp.*, *Peniophora lycii*, *Trametes pubenscens and Agaricus bisporus* [6,7]. The phytase was used in many areas such as animal feed, the food industry, the preparation of myo-

inositol phosphate, the paper industry, soil improvement and the elimination of environmental pollution. In recent years, some studies had been conducted, to find ways reduce the amount of phytate in cereal products.

In this study, the phytase enzyme was purified from edible, non-toxic Oakbug milkcap (*Lactarius quietus*) mushrooms using ammonium precipitation and DEAE-Sephadex ion-exchange chromatography techniques, then it was immobilized onto the surface of chitosan and modified with nano-magnetite to ensure prolonged usage. The industrial uses of pure enzymes, values of optimum pH, optimum temperature, stable pH, and stable temperature of soluble and immobilized enyzme were determined to make comparisons and optimization of the method. Measurements of the activity of soluble and immobilized enzymes were performed against different metal ions to determine the altered factors of enzyme activity. The their applicability of the degradation of phytate was then investigated in foodstuff cereal origin, using free and immobilized enzymes.

2. Materials and Methods

2.1. Chemicals

Chemical. Bovine serum albumin (BSA), Na-phytate, xylan, chitin, starch, gelatin, DEAE-sephedex, Sephacryl S-200, ethylene diamine tetra acetic acid (EDTA), dithioerythritol, β-mercaptoethanol, and agents for SDS-PAGE were purchased from Sigma (USA). Ethanol, sodium acetate (CH_3COONa), ammonium sulphate ($(NH_4)_2SO_4$), sodium chloride (NaCl), sodium hydrogen phosphate monohydrate (Na_2HPO_4 x H_2O), glutaraldehyde were purchased from Merck (Darmstadt, Germany). All other chemicals were of the analytical grade.

2.2. Plant Material and Storage Conditions

Oakbug milkcap mushrooms (*Lactarius quietus*) were collected from Manisa, Turkey and the surrounding area from May to July. They were stored at -40°C until studying. The mushrooms were identified by a botanist.

2.3. Purification of the Phytase Enzyme from Oakbug Milkcap Mushrooms (*Lactarius quietus*)

The phytase enzyme was purified from the mushrooms in two steps. In the first step, the ammonium sulfate precipation was performed in the homogenate of the mushroom in the range of 0-20%, 20-40%, 40-60%, 60-80% and 80-100%. The precipitate range with the highest activity was detected. Then, the precipitate was dissolved in 20 mM Na-acetate buffer at pH 5.5. It was then dialyzed against the same buffer [8,9,10].

In the second step, the homogenat was applied to the previously balanced with 20 mM of Na-acetate buffer (pH 5.5) and the DEAE -Sephadex column. The column was washed with the same buffer. Phytase was eluted from the column by increasing the ionic strength gradient of the buffer. The activity of the phytase enzyme was determined in the eluates using a Na-phytate substrate. The eluates which showed phytase activiy were combined with each other and the enzyme pool was created [9,10].

2.4. SDS-PAGE Electrophoresis

The Laemmli method was used to determine the number of subunits of the purified phytase from Oakbug milkcap mushrooms (*Lactarius quietus*) [11]. Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was performed in two different acrylamide concentrations with 3% stacking gel and 10% seperation gel.

2.5. Immobilization of the Purified Phytase Enzyme

2.5.1. Preparation of the Chitosan Particles

The method proposed by Chiu et al. [12] was used in the preparation of the chitosan particles. The chitosan was stirred with acetic acid for three hours. Then, the mixture was neutralized by adding 1 N NaOH in an ethyl alcohol medium. Chitosan molecules were then washed with distilled water. The moleculs were treated with a 5% glutaraldehyde solution for 4 hours to bind the amino group to the chitosan molecule. Finally, chitosan molecules were washed with distilled water under a vacuum to remove the non-binding glutaraldehyde from the medium.

2.5.2. Preparation of Nano-Magnetite Chitosan Particles and Immobilization of the Purified Phytase Enzyme

The magnetic property of the surface of chitosan particles was allowed by treating with dispersed Fe_3O_4 in distilled water. After treating, the chitosan with nano particles, the chitosan was washed with distilled water to remove the non-binding nano particles. Then, the wet precipitate was dried over s 72 hour time period at 40°C and it was stored at 4°C for use in experimental studies.

The purified phytase enzyme from Oakbug Milkcap (*Lactarius quietus*) was immobilized to the surface of modified with Fe_3O_4 NPs and activated with the glutaraldehyde chitosan in the Na-acetate buffer solution (pH 5.5), which contained 25 mM $NaCNBH_3$. The amount of protein was determined in the reaction medium at different time intervals, by using the Warburg and Bradford methods. The amount of the binding phytase enzyme was determined against time.

2.5.3. Determination of the Optimum Conditions for the Immobilization of the Purified Phytase Enzyme Onto the Modified Chitosan with Fe_3O_4 NPs

The following optimization procedure were performed to determine the most suitable immobilization conditions of the phytase enzyme to support material for all studies of immobilization.

The most appropriate immobilization pH was determined mading immobilization in the pH range of 5.0 to 8.0. The most appropriate immobilization temperature was determinated by carrying out immobilizations at 10°C, 15°C and 20°C in a previously determined pH.

The phytase binding efficiency (E) is defined as follows:

$$E = (C_1 - C_o) / C_1$$

where C_1 and Co are the amounts of phytase protein in the solution before and after immobilization, respectively. The activity amount of the immobilized phytase was calculated as follows:

$$\text{Immobilized phytase} = A/(A_1 - A_o)$$

where A is the activity of the immobilized phytase and A_1 and A_o are the activities of the free phytase in solution before and after immobilization, respectively.

2.6. Determination of the Phytase Activity

Phytase activity was determined by measuring the amount of inorganic phosphate which was hydrolyzed from sodium phytate. The amount of phosphate hydrolyzed in the medium was determined by assaying the changes of absorbance at 700 nm [13]. One unit of phytase activity (1 EU) was defined as the amount of enzyme that released 1 μmol inorganic phosphate at optimum conditions in a one minute reaction.

2.7. Protein Determination

The protein concentration of the samples was determined spectrophotometrically using the Bradford method [14]. Bovine serum albumin (BSA) was used as a standard.

2.8. Determination of the Optimal pH for Activity and Stability

The optimum pH was determined at 50°C using different buffers: 50 mM citric acid buffer for pH 3.0-5.0, 50 mM MES buffer for pH 5.0–7.0, and 50 mM Tris buffer for pH 7.0-9.0. The immobilized and free-form phytase were suspended in each buffer at different pH levels to give an activity of 30 U/mL. The pH stability was tested in 100 mM buffer incubated for 4 h at 4°C.

2.9. Determination of Temperature Optima and Thermal Stability

The various temperatures (20-90°C) were tested in 50 mM Na-acetate buffer (pH 5.0) to determine the optimal temperature for the enzyme reaction. To determine the thermostability of the enzyme, thermal challenges at different temperatures (60°C, 70°C, 80°C, and 90°C) were performed in 50 mM acetate buffer (pH 5.0). Immediately afterwards, the heat-treated enzymes were place on ice. Residual phytase activity was measured at 50°C and pH 5.0, as described previously [14]. Each treatment was done a minimum of three times.

2.10. Effect of Some Metal Ions

The influence of various metal ions such as Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} at concentrations of 1 and 5 mM on enzyme activity was investigated by preincubating the phytase with different compounds for 10 min at room temperature. Residual activity was calculated against control.

2.11. Hydrolysis of Phytate in Some Cereals

The legumes were collected at local markets such as green lentils, red lentils, peas, pinto beans, beans, brass, corn, dried corn, oat, rye, wheat, broad bean, chicpes and peanuts were used for hydrolyzing the phytate. First of all, the cereals were washed elaborately; then they were kept in air to dry, and they were homogenized. Into the 5 g legume sample homogenate, 2 mL free enzyme (30 U/mL) solution was added. Also, the same experimental mixture was created for immobilized phytase enzyme. At 5.0 pH value, the legumes homogenates were processed with free phytase and immobilized phytase enzyme for 4 hour at 50°C. Then, the amount of free phosphorus was determined by assaying the standart activity against blank sample which was consisted of distilled water [14]. The amount of phosphorus was calculated by using calibration curve. A control was prepared without added enzyme for all the assays.

3. Results and Discussion

Phytases (myo-inositol hexakisphosphate phosphohydrolase) [EC 3.1.3.8] belong to the class of fosfomono esterase of enzymes and thwy are enzyme which catalyze hydrolysis reaction to phosphate, inositol and phosphate result in sequentially hydrolysis groups of organophosphate of phytic acid [15,16].

3.1. Purification and Characterization of Phytase from Oakbug Milkcap (*Lactarius quietus*)

The phytase enzyme was purified in three steps including ammonium sulfate precipitation, DEAE-sephadex ion exchance chromatograhy and Sephacryl S200 gel filtration chromatography from Oakbug Milkcap (*Lactarius quietus*). All purification steps were summarized in Table 1.

Table 1. The purification process of purified phytase enzyme from Oakbug Milkcap (*Lactarius quietus*)

Enzyme Fraction	Volume	Activity	Total Activity		Protein	Specific Activity	Purification
	mL	EU/mL	EU	%	(mg/ml)	EU/mg protein	Fold
Crude extract	50	1952.6±0.33	$9.8x10^4$	100	195.0±1.13	10.01	-
$(NH_4)_2SO_4$ (40-80%)	30	1413.1±0.71	$4.24x10^4$	43.3	18.6±0.25	75.97	7.58
DEAE-sephadex	30	1016.1±0.22	$3.05 x10^4$	31.1	3.12±0.12	325.7	32.54

In the first stage, the crude extract was precipitated between the range of 0-100% ammonium sulfate saturation. These results showed that phytase enzyme activity was determined the highest rate in the range of 40-80% saturation by precipitation. The purified phytase in 43.3% yield and 7.58-fold purification was applied to a DEAE-sephadex ion exchange column.

In the second step, amonyum sulphate fraction of 40-80% was applied to the DEAE- sephadex ion exchange column. The phytase was purified more than 32.54 times fold with a yield of 31.1%, and its specific activity was eqivalent to 325.7 EU/mg protein (Table 1). Phytase enzyme from Oakbug Milkcap (*Lactarius quietus*) mushroom was purified with anion exchange chromatography and it was presented in Figure 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)'s results were presended for purified the phytase enzyme, 45 kDa consists of one subunit has been identified (Figure 2).

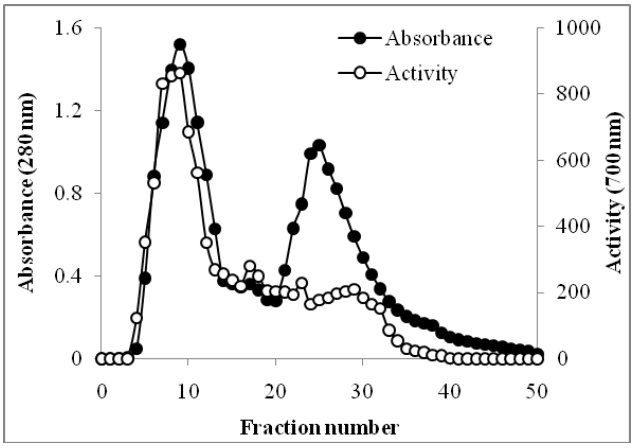

Figure 1. Purification of phytase by ion exchange chromatography using DEAE-sephadex

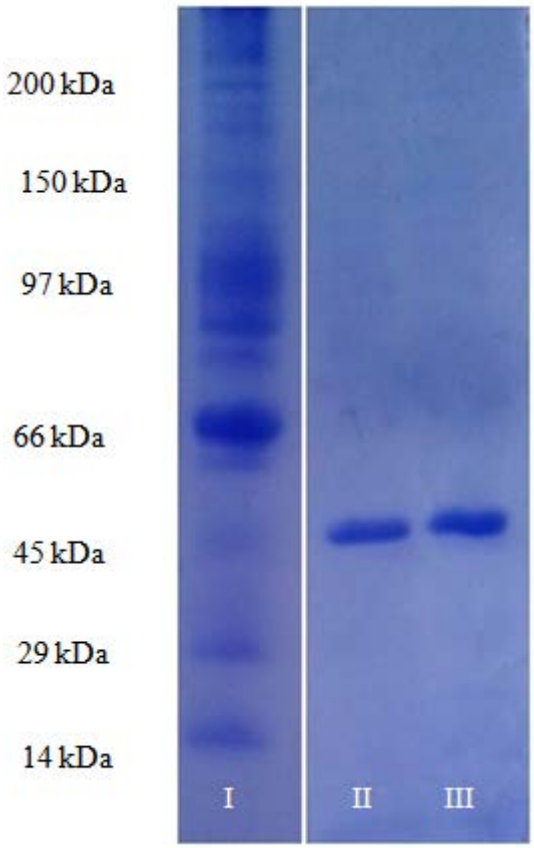

Figure 2. SDS-PAGE electrophoretic pattern of phytase [purified phytase enzyme from Oakbug Milkcap (*Lactarius quietus*)

Standart protein (β-Amylase, sweet potato, 200 kDa; alcohol dehydrogenase, yeast, 150 kDa bovine serum albumin, 97.4 kDa; rabbit muscle phosphorylase A, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 29 kDa; carbonic anhydrase); cytochrome c, horse heart 14 kDa (I); Oakbug Milkcap (*Lactarius quietus*) (II and III)].

To find the molecular weights of the active form of the purified enzyme, Sephadex G-100 gel filtration chromatography was performed and the same results were obtained. Also, molecular weight of thermostable monomeric phytase enzyme purified from the *Mycobacterium smegmatis* and *Escherichia coli* was found around 45 kDa [17,18].

Firstly, chitosan was activated by binding the glutaraldehyde to the amino group (-NH_2) located chitosan for connecting the purified phytase enzyme to the modified chitosan molecules with F_3O_4 nano particles. Then, it was provided binding of purified phytase by creating schift base to glutaraldehyde. $NaCNBH_3$ was used for reducting base of schift formed in both of two steps. After synthesized the support material, its surface was coated with Fe_3O_4 nanoparticles for purpose gain magnetic feature of chitosan. Made reactions were shown below (1-3).

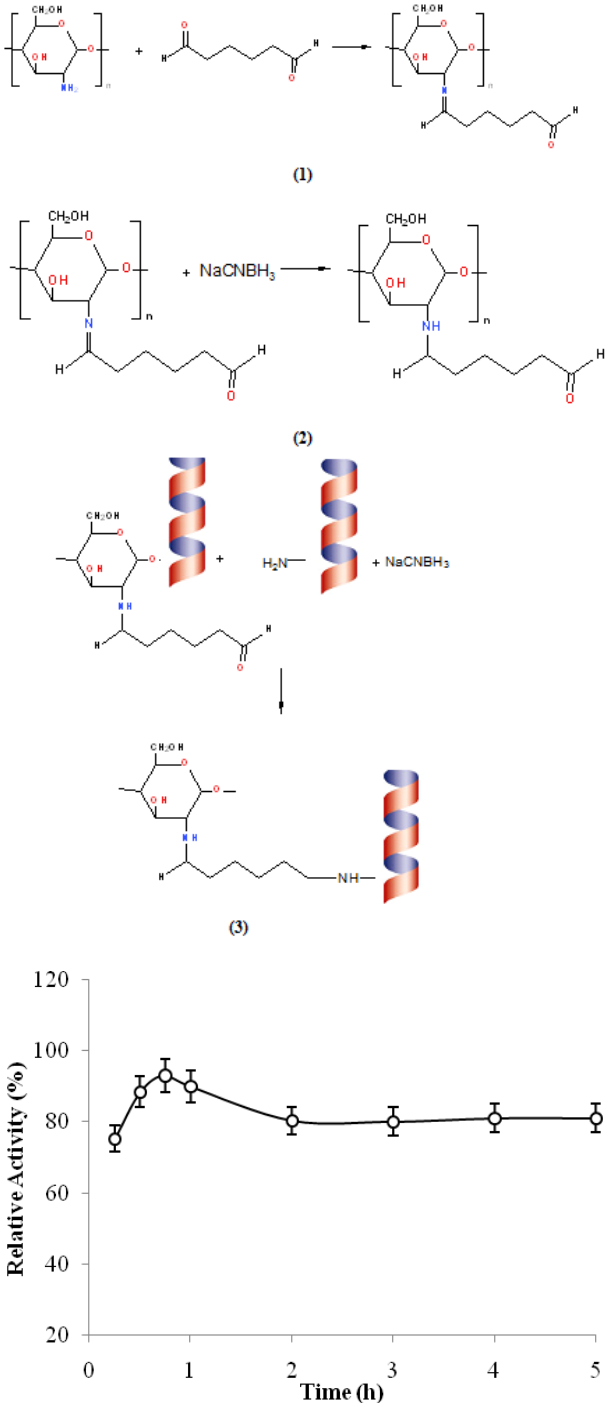

Figure 3. The effect of time to the immobilization of phytase enzyme onto modifed chitosan with nano magnetite

pH and temperature was primarily determined in order to determined the optimum conditions of covalent immobilization to the surface of magnetized citosan with nano Fe_3O_4 of pure phytase enzyme. Pure phytase enzyme were immobilized by using appropriate buffers among pH

4-8, and it was calculated relative activity (%) of immobilized phytase. Accordingly, it was determined that the pure phytase enzyme was connected to support material at the high level at pH 5 and 20°C. Then, the immobilization was monitored for 5 hours to determine the most approiate immobilization time at pH 5 and 20°C. It was determined that purified phytase enzyme was connected to magnetized citosan with nano Fe_3O_4 support material after 1 hour at the rate of 82.5% (Figure 3).

3.2. Structural Characterization of Support

Structure of chitosan, modified chitosan with nano magnetite and purified phytase immobilized modified chitosan with nano magnetite at SEM images were shown in Figure 4a-c. The thickness of the wall is about 20 ~ 30 nm, the pore size is about 0.5 ~ 1 μm. From the SEM image, it was obseved that the support material was fine-pore structure and its structure was appropriated to modify with nanomaterials and to immobilization with phytase enzyme. In addition, it had a large-sized support and structural stability.

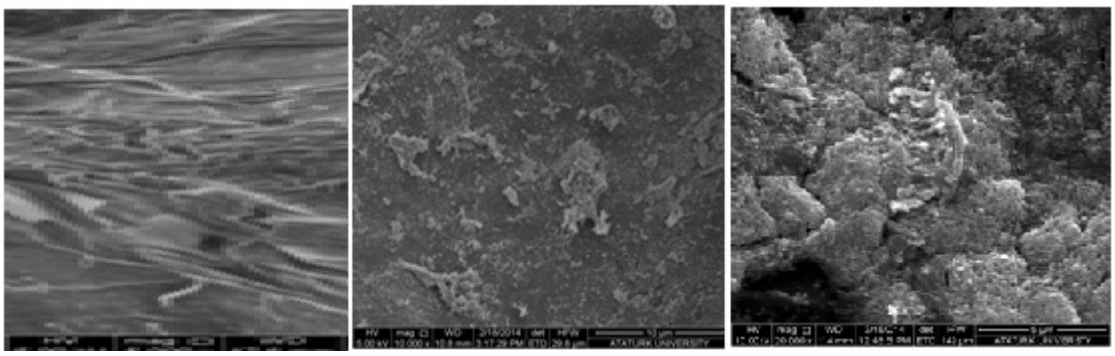

Figure 4. SEM images I: nature chitosan; II: modified chitosan with nano magnetite; III: purified phytase immobilized modified chitosan with nano magnetite

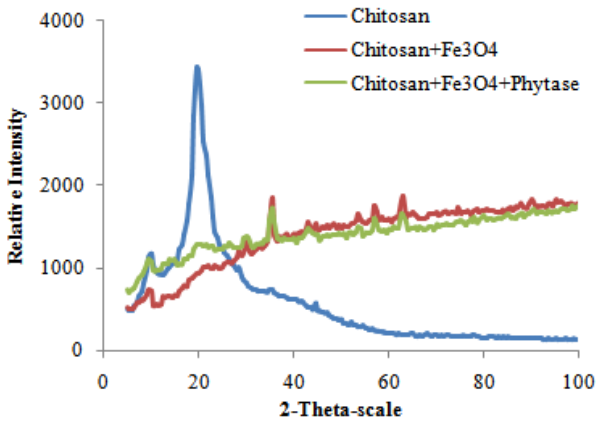

Figure 5. XRD photographs for nature chitosan; modified chitosan with nano magnetite; purified phytase immobilized modified chitosan with nano magnetite

3.3. X-Ray Diffraction (XRD) Analysis

Figure 5 shows XRD patterns of nature chitosan; modified chitosan with nano magnetite; purified phytase immobilized modified chitosan with nano magnetite. XRD patterns chitosans are illustrated in Figure 5. The XRD pattern of chitosan exhibits broad diffraction peaks at 2θ = 9,5° and 19,5° which are typical fingerprints of crystal chitosan [19]. In the XRD pattern of prepared Fe_3O_4 modified chitosan NPs and purified phytase immobilized modified chitosan with nano magnetite, the peak corresponding to chitosan could be seen at around 2θ = 20.0°, but it became much lower and wider. Also, five diffraction peaks were observed at 2θ = 35.5°, 44.5, 54.5, 57.5°, 63.0°. These peaks are belong to typical fingerprints of Fe_3O_4 NPs [20].

3.4. FTIR Analysis

The structure of the support material of nature chitosan (a), modified chitosan with nano magnetite (b), immobilized magnetic Fe_3O_4-chitosan nanoparticles with purified phytase from Oakbug milkcap (*Lactarius quietus*) (c) was confirmed by FTIR analysis. The spectra of chitosan showed a broad absorbance at 3650.60 - 2879 cm^{-1} (O-H, hydroxyls and NH stretching vibrations of free amino groups), at 2879.93 cm^{-1} and 2116 cm^{-1} (-CH_3 and -CH_2 in chitosan structure), at 1638.38-1029.96 cm^{-1} (-C=O stretching of amide, -C=O secondary amide, -N-H bounds stretching vibrations) and at 891.38 cm^{-1} and 608.38 cm^{-1} (-C-H bending of amide bonds stretching vibrations) in curve Figure 6a-c. In the Figure 6b and 6c, the peaks at 557.92 cm^{-1} and 554.45 cm^{-1} related to Fe-O group of Fe_3O_4 nanoparticles. When the Figure 6a compared with Figure 6b, a new sharp peak appeared at the 1627 cm^{-1}. It indicated that at the 2116.66 cm^{-1} peak of N–H bending vibration shifted to 1589.68 cm^{-1} and chitosan react with glutaraldehyde to form *Schiff* base [21,22]. It was shown that there was no significant change

in the functional biomass groups of magnetic Fe_3O_4-chitosan nanoparticles after immobilization of phytase enzyme. The results of FT-IR spectrums indicated that chitosan was coated to the magnetic Fe_3O_4 nanoparticles and phytase enzyme was immobilized onto the magnetic Fe_3O_4-chitosan nanoparticles successfully.

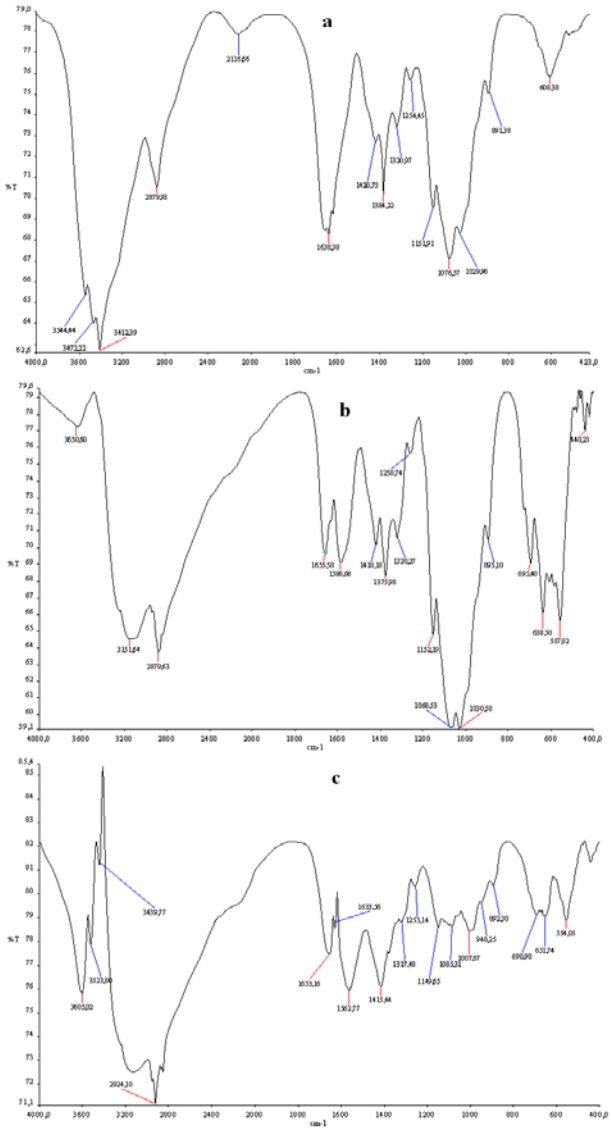

Figure 6. FT-IR spectrum of nature chitosan (a), modified chitosan with nano magnetite (b), immobilized chitosan with purified phytase from Oakbug milkcap (*Lactarius quietus*) (c)

3.5. Biochemical Properties of Free and Immobilized Phytase

3.5.1. Loading Amount and Activity of Immobilized Phytase

The average loading amount of modified support is about ng/g. The phytase enzyme was bound to surface as covalent, it was also bound there with weak bonds with wander walls bonds and binding rate was increased. In a study, it was observed that the enzyme was connected to modified support using nanostructure at most amount and powerfully. This research was supported our work [23].

3.6. Effect of Incubation Time, pH and Temperature

3.6.1. Effect of Reaction Temperature

The reaction was carried out from 10 to 90°C using Na-fitat as a substrate at pH 5.0 for 30 min incubation time for free and immobilized phytase enzyme. The maximum phytase activity for free and immobilized phytase was found at 50°C. But, it was determined that both their activity were decreased in higher temperatures (Figure 7a.).

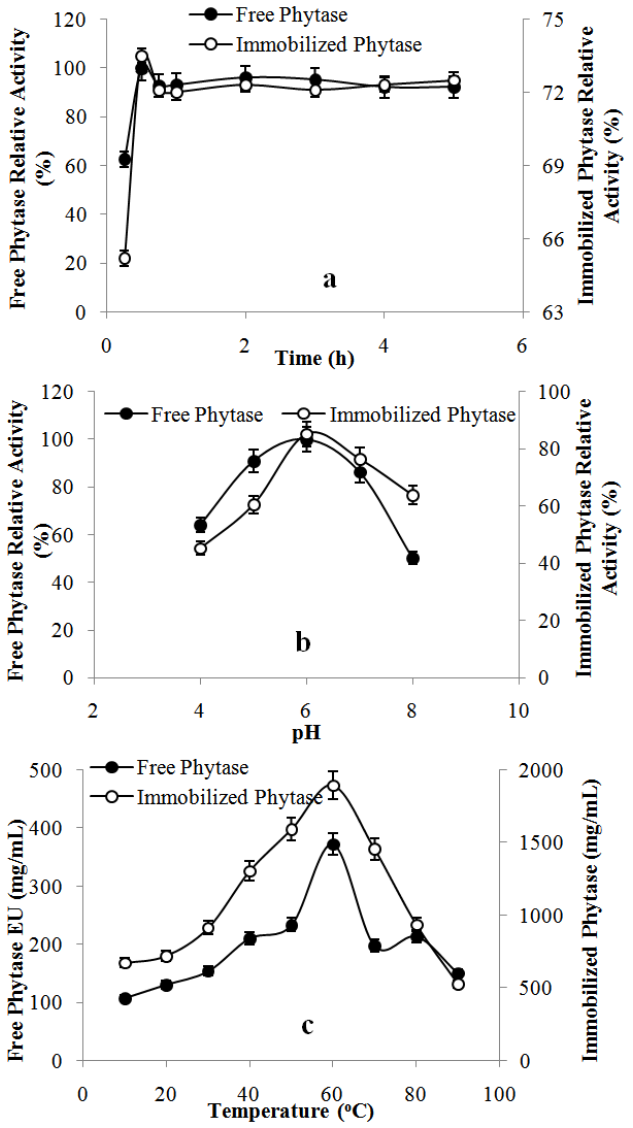

Figure 7. Optimization of purified and immobilized phytase enzyme activity. Reaction condition for a reaction time optimization (temperature 60°C and pH 6.0), b pH optimization (temperature 60°C and incubation time 30 min), and c temperature optimization (pH 6.0 and incubation time 30 min)

3.6.2. Effect of pH

Enzyme substrate reaction was done at 50°C for 30 min varing the pH from 4.0 to 8.0. Optimum pH was found as 5.0 for free and immobilized phytase enzyme (Figure 7b).

3.6.3. Effect of Reaction Time

Enzyme substrate reaction was done at 50°C and pH 5.0 during 5 h. For free and immobilized phytase, maximum enzyme activity was found to be at 45 min incubation time and and reaction was reached to balance at 1 hour (Figure 7c).

3.7. pH Stability and Termal Stability

3.7.1. Thermal Stability

Enzymes are composed of protein and they are often sensitive to heat and they can be inhibited. Immobilized enzymes were resistant to high temperatures, so it had been many advantages for use in applications. The thermo-stability of the immobilized phytase was measured in comparison with free phytase as shown in Figure 8a. The rates of thermal inactivation of the soluble and immobilized enzyme were studied at the temperature range of 20-90°C at pH 5.0 in 50 mM Na-acetate buffer.

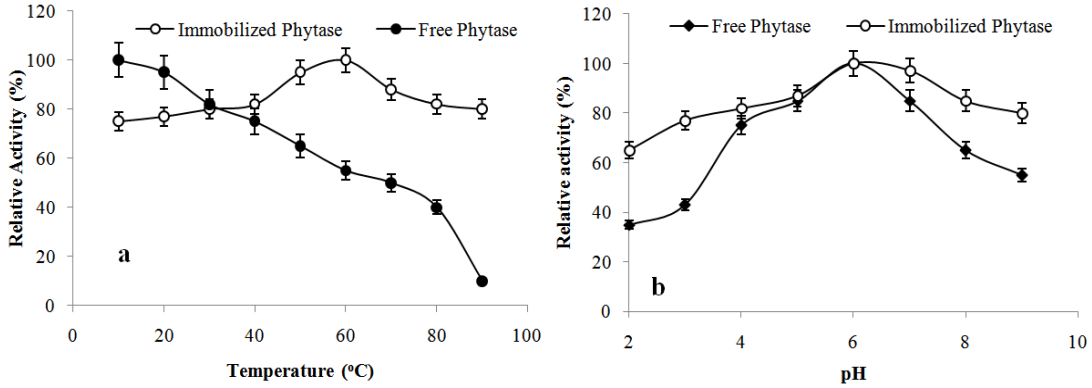

Figure 8. (a) Thermal stability, (b) pH stability for free and immobilized phytase

While free enzyme was losting its activity at approximately rate of 60%, at 80°C after heat treatment for 1 hour, immobilized phytase was only losted its activity at 38% rate. Consequently, covalently linked immobilized phytase onto Fe_3O_4 modified chitosan NPs was more resistant to temperature than soluble phytase. When enzyme was covalently bounded onto modified chitosan with nano magnetite, it could be thought that phytase enzyme was protected from environmental factors which was caused conformational changes.

3.6.1. pH Stability

Free and immobilized phytase enzyme were incubated for 1 hour at 4°C by using buffer of acetate from pH 3 to 5, phosphate from pH 6 to 7 and Tris/HCI from pH 8 to 9 and their activity were measured after 1 hour and the results are shown in Figure 8b. It was determined that free enzyme was maintained 43% rate of its activity while immobilized enzyme was maintaining 77% rate of its activity at pH 3. Also, free phytase was maintained 55% rate of its activity while immobilized phytase was maintained 80% rate of its activity at pH 9.0. As seen from the results, immobilized phytase enzyme activiy was more stable againts different pH values according to free enzyme.

3.8. The Effects of Fe^{2+}, Cu^{2+}, Hg_2^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} on Activity of Free and Immobilized Phytase Enzyme

Effects of Fe^{2+}, Cu^{2+}, Hg_2^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} on activity of free and immobilized purified phytase enzyme from Oakbug Milkcap (*Lactarius quietus*) mushroom onto nano-Fe_3O_4-MD CTS were investigated and all results were given in Table 2 as relative activity%. While all metal ions were higly inhibited to the activity of free phytase enzyme, only Co^{2+} was higly inhibited immobilized phytase enzyme. While Hg_2^{2+} and Mg^{2+} ions were increased the activity of immobilized phytase, Fe^{2+}, Cu^{2+} and Zn^{2+} aproximately had no effect it. From the results, it was concluded that immobilized phytase enzyme was more resistant to metal ions. Because of this feature of enzyme, immobilized phytase could be found to be highly suitable for industrial application.

Table 2. The effect of some chemical compounds on free and immobilized phytase activity

Chemical Compounds	Concentration (mM)	Free Phytase Relative Activity (%)	Concentration (mM)	Immobilized Phytase Relative Activity (%)
None	-	100 ± 0.0	-	100 ± 0.0
Fe2+	1	70.3 ± 2.01	1	89.3 ± 1.25
Cu2+	1	43.3 ± 2.3	1	81.1 ± 1.02
Hg22+	1	105.4 ± 1.1	1	116.8 ± 0.3
Co2+	1	72.97 ± 1.4	1	43.3 ± 1.15
Mg2+	1	59.5 ± 1.13	1	113.5 ± 1.7
Zn2+	1	78.4 ± 0.14	1	86.8 ± 0.6

3.9. Hyrolysis of Phytate in Some Cereals

Phytic acid is capable of making strong chelating. The phytic acid is forming insoluble complexes salts by combined with metal cations (Zn^{+2}, Cu^{+2}, Co^{+2}, Mn^{+2}, Ca^{+2} ve Fe^{+2}) and they prevent metal ions' absorbation from intestinal. Phytates decrease digestibility of protein and amino acid and they lead to negative effect in mineral intake as a result of forming phytate-protein complex which was harder disintegrating by proteolytic enzymes binding endogenous proteases such as especially trypsin and chemotrypsin [24,25]. Therefore, it could be caused iron, calcium and zinc deficiency especially in communities of adopt the type of feeding vegetative [26].

$$\text{Phytic acid} \xrightarrow{\text{Phytase}} \text{Inoganic monophate}$$
$$+\text{Myo-inositol phosphate}+\text{Free myo-inositol}$$

Due to this reason, phytic acid which was located in the outer shell of grains and cereals was hydrolyzed using free and immobilized phytase enzyme and the results were

given in Table 3. From the results, it was observed that immobilized phytase enzyme was more effective at the hydrolysis of phytate in dry beans and it was showed the highest effective on beans, wheat and peanuts at the end of 4th as rates of 75.2, 77.8 and 76.7%, respectively. Immobilized phytase enzyme could be used as additive material in bread making, preparation of vegetable protein hydrolyzate products and breakdown of bran in cereals.

Table 3. The hydrolsis of phytate from some legumes using free and immobilized phytase enzymes

		Free Phytase Activity		Immobilized Phytase Activity	
	Time (h)	2h	4h	2h	4h
CEREALS	Green Lentils	26.74 ± 1.21	43.7 ± 1.13	38.8 ± 0.28	56.5 ± 0.51
	Red Lentils	21.0 ± 0.01	31.93 ± 1.5	28.3 ± 1.45	53.2 ± 0.18
	Peas	35.9 ± 0.52	57.0 ± 0.32	38.2 ± 0.22	62.5 ± 1.3
	Pinto Beans	53.13 ± 0.2	59.44 ± 0.06	63,5 ± 0.7	68.7 ± 0.51
	Beans	56.76 ± 1.16	61.75 ±1.0	68,28 ± 1.05	75.2 ± 0.15
	Brass	18,29 ± 1.4	27.82 ± 2.13	33.7 ± 1.7	47.6 ± 1.31
	Corn	29.01 ± 0.15	40,68 ± 1.14	33,3 ± 0.22	58.4 ± 2.2
	Dried Corn	19.34 ± 0.15	28.2 ± 0.36	25.05 ± 0.03	38,7 ± 1.13
	Oat	20.73 ± 1.17	32.2 ± 0.31	26.7 ± 0.36	42.3 ± 1.6
	Rye	29.38 ± 4.31	41.4 ± 2.1	35.1 ± 0.13	56.8 ± 1.26
	Wheat	60.67 ± 0.3	63.21 ± 0.6	63.28 ± 0.28	77.8 ± 1.02
	Broad Bean	44.45 ± 0.4	51.34 ± 1.4	45.8 ± 1.6	58.7 ± 0.22
	Chickpea	33.93 ± 0.15	52.29 ± 0.03	36.4 ± 0.01	58.1 ± 1.3
	Peanuts	48.66 ± 1.9	67,99 ± 0.07	65.6 ± 0.27	76.65 ± 1.05

4. Conclusion

The phytase was firstly purified and characterized from Oakbug Milkcap (*Lactarius quietus*) and immobilized onto modified citosan with magnetite. Purified and immobilized phytase enzyme were found that it had high catalytic activity and it was resistance to metal ions, high temperature and different pHs. Also, it was observed that immobilized enzyme was effective on phytate in dry beans. Due to this reason, the immobilized phytase enzyme onto surface of nano-Fe_3O_4-MD CTS could also be used in different industrial areas. This research was focused on the food industry. In this context, it could be had a huge potential for use in the field of food, feed and fertilizer.

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