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Sorption of ruminal fluid derived phytase onto modified nanoparticles and determination of its desorption capacity

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Abstract

Ruminal fluid of slaughtered cows was used as a free phytase source. Magnetic nano-particles (MNP_s) were grafted with allyl 2,3-epoxypropyl ether, subsequent by coupling with cibacron blue. Cibacron blue as the main monomer is a known ligand for enzyme sorption. Characteristics of the coated MNP_s were proved using Fourier transform infrared spectroscopy methods, transmission electron microscopy, and thermogravimetric analysis. These results were then compared with those of non-modified MNP_s. Phytase which derived from clarified rumen fluid was then adsorbed at different pH, shaking time, and various levels of temperature via ionic bound. The best adsorption efficiency was recorded under conditions of pH 7, temperature of 6 °C, and shaking time of 60 minutes. Desorption phase was performed in two different shaking times and four different concentrations of NaCl solution. The highest percentage of desorption was lower than 20%, which achieved at 1 M NaCl solution in 90 minutes of shaking ($P < 0.05$).

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Introduction

Enzymes are efficient biocatalysts extensively used in different technologies. These protein or protein based substances are yet difficult to purification, which makes them expensive (Caterina *et al.*, 2013). Since enzymes are proteins, they are susceptible to denaturation by digestive enzyme or other factors, which can alter their structure. Enzymes have ideal temperature, pH, and some other conditions, where they function are more readily (Purva and Uttam, 2004). Phytases are chemically recognizes as myo-inositol-1-6-hexakisphosphate phosphohydrolase, and catalyze the stepwise liberate of phosphate (Debnath *et al.*, 2005). The amount of phytase required for releasing one µmol orthophosphate from phytin per minute at pH 5.5 and 37 °C is known as one unit of phytase activity (Zyla *et al.*, 1995). Phosphorus is one of the most expensive components of commercial poultry feeds. Thus, it is necessary to optimize the utilization of phosphorus with exogenous phytase to meet a lower feeding cost by poultry (Bedford, 2000). In plant derived foods, phytic acid acts as an anti-nutritional factor, thus phytase not only liberates the phosphorus from poultry diets but it also increases the availability of calcium, magnesium, protein and lipid in poultry feeds. As a consequence by releasing phosphorus bound in plant based diets, phytase makes more phosphorus available to promote the rate of weight gain and bone growth (Baruah, 2007; Harland and Morris, 1995). Ruminants (cattle and goat), opposite of mono gastric (poultry and horse), utilize the phosphorus in phytate form. Therefore, rumen fluid is an appropriate source of microbial phytases which having temperature and pH stability, high metal ion resistance, and suitable specific activity for supplementation of animal feed and inositol production (Chwan *et al.*, 2004). Because immobilization/sorption is an extremely efficient method for improving enzyme stability, the immobilization/sorption of these biocatalyst molecules (responsible for the thousands of metabolic processes) onto inorganic support is required for industrial application (Khoshnevisan *et al.*, 2011; Liao and Chen, 2001). The two main procedures for enzyme immobilization/sorption on different types of support can be separated into chemical and physical method by covalent bond formation (between enzyme and carrier) and entrapment on a solid support (adsorption of the enzyme on material), respectively (Aksoy *et al.*, 1998). Magnetic nanoparticles (MNPs) have been used as supports for sorbed enzymes over the past few years. MNPs as the enzyme sorbent have many of the main advantages, such as the potential to improve sorption activity recovery, enzyme features, provision of separation, repeated usage, greater specific surface area and lower mass convey resistance and, less co-precipitation (Halling and Dunnill, 1980; Tien and Chiang, 1999; Talekar *et al.*, 2012; Zhou *et al.*, 2013). The sorption of several enzymes onto magnetic nanoparticles has been investigated by some researchers. Nevertheless, based on our knowledge there is no reports of phytase sorption on MNPs.

The point of this investigation was to evaluate the feasibility of phytase separation and purification by sorption onto modified MNPs. In this study, magnetic (Fe_3O_4) nanoparticles were coated with cibacron blue F-3GA as the functionalized ligand. The modified magnetic nanoparticles (MNPs) were applied to adsorb and desorb of phytase. Clarified rumen fluid (CRF) was used as a low-cost source of enzyme pool. All adsorption process steps were optimized at various levels of temperature, pH, and shaking times. Desorption steps were accomplished using a NaCl solution in different concentration. Ultimately the NaCl residue was removed by a dialyzing method, which increases enzyme concentration because of the selectively permeable membrane of dialysis bag.

Materials and methods

Materials

Allyl 2,3-epoxypropyl ether, 3-(trimethoxy silyl)-1-propanethiol, bovin serum albumin fraction V, *N,N*–dimethylformamide, acetic acid (glacial), $FeCl_2 . 4H_2O$, and $FeCl_3 . 6H_2O$ were purchased from Merck Millipore (Darmstadt, Germany). Cibacron blue F-3GA (FLUKA), coomassie brilliant blue R 250 (SIGMA), and sodium phytate were purchased from

SIGMA-ALDRICH (USA). 1,4–Dioxin was purchased from CHEM-LAB (Belgium).

Apparatus

Separation of rumen fluid from digesta was carried out by laboratory centrifuges (Sigma–3K 30, Germany). An orbital shaker (B. Braun Biotech, USA) was used to stir CRF and coated MNPs. The characteristics of Modified MNPs were compared to bared MNPs using Fourier transform infrared spectroscopy (FT-IR) (Thermo Nicolet, NEXUS 870 ESP, USA). The physical properties of coated MNPs were checked using the thermogravimetric analysis (TGA) by Simultaneous Thermal Analyzer (SCINCO Model 1500, Korea). The morphology of prepared MNPs were measured by transmission electron micrographs (TEM) (Philips EM 208 electron microscope, 100 KV grid cu 200 Mesh, former Czechoslovakia). Enzyme activity was determined using spectrophotometric method (Varian Cary 50 Bio UV/Vis spectrophotometer, USA).

Preparation of support

Magnetic nanoparticles (MNPs) were synthesized by dissolving 3.97 g $FeCl_2$ $4H_2O$ and 2.3 g $FeCl_3$. $6H_2O$ in 100 mL deionized water under nitrogen gas with vigorous stirring at 1000 rpm (Mahdavian and Mirrahimi, 2010). Then 7.5 mL of ammonia (NH_3) solution was added stepwise over a period of 10 minutes, simultaneously. Consequently, chemical precipitation was prepared under anaerobic conditions at 85 °C to obtain 2 g of iron oxide (Fe_3O_4). After 2.5 hours, the magnetic precipitation was separated from the solvent by magnetic decantation and rinsed with deionized water twice, and once with ethanol to remove reaction residues and water respectively. Finally, the prepared MNPs were evaporated to dryness.

Modification of MNPs

After successful synthesis of MNPs, surface modification was accomplished which involved mixing 4 g of prepared MNPs with 3-(trimethoxy silyl)-1-propanethiol (2.5mL) and dioxin (50 mL) for 3 days at 80 °C in a 150 mL three-necked balloon under nitrogen gas and with magnetic stirring. The obtained MNPs were refluxed with 20 mL ally 2,3-epoxy propyl ether, 50 mL *N,N*-dimethylformamide and 0.1 g 2,2′-Azobis(2-methylpropionitrile) under Nitrogen gas. Magnetic stirring was performed for 8 hours, simultaneously. At last, the surfaces modification followed by coupling with cibacron blue. Cibacron blue F-3GA (1 g), 50 mL NaCl solution and 20 mL *N,N*-dimethylformamide was mixed under vigorous stirring at 24 °C for two days. Preparation was continued by washing up modified MNPs with distilled water several times until the water run clear and colorless. TEM, FTIR, and TGA were used for characterization of modified MNPs. The methodology used for preparation of the grafted MNPs is explained in Figure 1.

Preparation of sample

Equal amounts of the rumen contents of 8 slaughtered cows were collected to create a homogenous pool sample. The pool sample was mixed and transferred to the laboratory in thermos flasks. The obtained ruminal fluid mixture was centrifuged at 8000 × *g* for 20 minutes to separate digesta from CRF. Whatman filter paper No. 4 was used for the filtering of the residue supernatant of CRF under suction. Pool sample was divided into six different equal parts as treatments. The Phytase activity was determined by measuring the amount of released pi from sodium phytate (Kim and Lei, 2005). One unit of phytate activity was defined as the amount of enzyme required to release 1 µmol pi per minute under the assay condition (Hubel and Beck, 1996). The specific activity (S.A) was explained in units of enzyme activity per mg total protein.

Phytase adsorption and desorption

The desorption of phytase was carried out by mixing 7 mL CRF with 0.04 g modified MNPs at different contact time (10-60 minute), pH of source (5-8), and shaking temperature (6-45 °C). The 7 mL of CRF without any sorbent at similar experimental condition of the previous samples was examined such a control treatment. NaOH 0.5 M and citric acid 1 M solution were used to apply different pH values. The sorbed

phytases (sorbed phytase onto modified MNPs) were simply separated from CRF by an external magnetic field. The sorbed enzyme (defined as θ) was measured by differences between phytase activity of the samples and control treatment. Control treatment was considered without any sorbent at the similar condition. Protein concentration was calculated according to the Bradford (1970) procedure using bovine serum albumin as the standard protein. The different pool samples were investigated for protein concentration by spectrophotometer at 540 nm. The specific activity of phytase was determined as follow:

$$\text{Unit of activity } (IU/mg) = \frac{\text{activity of phytase } (IU/mL)}{\text{total protein(mg/mL)}} \quad (1)$$

Activity of phytase was calculated by this equation:

$$\text{Phytase activity } (IU/mL) = \frac{\text{released Pi (micromoles)}}{\text{mg enzyme in reaction} \times 1 \text{ min at } 40^{\circ}\text{c}} \quad (2)$$

The enzyme desorption process was established by mixing modified MNPs–phytase and 7 mL NaCl solution (0, 0.1, 0.5, and 1 M) at room temperature (Sutkeviciute and Sereikaite, 2008) with two different shacking times (15 and 90 minutes). Magnetic decantation was used to collect the remaining liquid of pool samples. The sorbed phytase onto coated MNPs were desorbed by NaCl solution. Separation of solved NaCl from free phytase was completed by dialyzing procedure (24 hours at 6 °C). The desorbed phytase activity was measured as follows:

$$\text{Apparent Desorption Percentage (ADP)} = \frac{\text{desorped enzyme activity } (IU/mg)}{\text{phytase activity in perliminary source } (IU/mg)} \times 100 \quad (3)$$

$$\text{True Desorption Percentage (TDP)} = \frac{\text{released phytase activity in dialysed liquid } (IU/mg)}{\text{sorbed enzyme activity } (IU/mg)} \times 100 \quad (4)$$

$$TDP = \frac{\text{desorped enzyme activity } (IU/mg)}{\text{sorbed enzyme activity } (IU/mg)} \times 100 \quad (5)$$

Statistical analysis

Adsorption data were considered with eight treatments for pH (5, 5.5, 6, 6.5, 7, 7.35, 7.5, and 8), three contact time (10, 30, and 60 minute), and four temperature of shaking time (6, 18, 24, and 45 °C). Desorption data were investigated with four treatments for different concentrations of NaCl solution (0, 0.1, 0.5, and 1 M) and two shaking time (15 and 90 minute). Each treatment had 6 replicate. Statistical analyses were conducted by complete randomized design (CRD). Value of $P < 0.05$ was considered significant. The means were compared by Duncan's multiple range tests. All above analyses were carried out by SPSS software versions 20.

Results and discussion

Characterization of sorbent

TEM micrographs

The particle size and morphology of coated and naked MNPs was investigated using TEM images. The diameters of naked and modified MNPs are explained as average values. As are shown in Figure 2, naked and modified MNPs have spherical shape with 20-80 nm diameters.

Analysis of FTIR

Modification of naked MNPs was considered by FTIR technique. The infrared spectrum of naked MNPs was compared with modified ones. The peak at 569 cm^{-1} in naked MNPs is ascribed to FeO groups in nano-particles. The further peaks at 2138, 1726, 1436, 1145, and 1240 cm^{-1} referred to aliphatic C-H, C=O, CH_2, SiO and C-O, respectively. These results indicate that modification of MNPs was performed successfully, which are shown in Figure 1.

Evaluation of naked and coated MNPs by TGA procedure

TGA was used as a technique to evaluate the thermal stability of naked and modified MNPs. The weight losses for naked and coated MNPs were 2.71% and 3.25% at 266 °C respectively, because of adsorbed water evaporation in both kind of nano-sorbent. Significant differences of weight losses in 400 °C between naked and modified MNPs were recorded. The percentage of weight loss for bared MNPs was 5.12 due to remove of water molecule in Fe_3O_4 structure. In other hand, 35% weight loss of coated MNPs at 400 °C is because of both withdrawal water molecule in structure of Fe_3O_4 and disintegration of

grafted polymer chain. This results shows that approximately 30% of organic chain (containing cibacron blue) was attached to inorganic part.

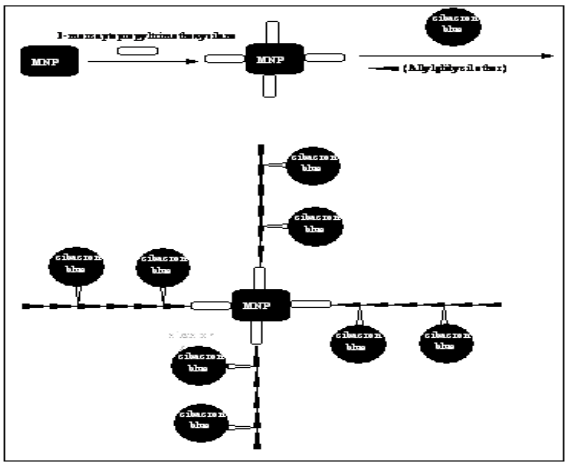

Fig. 1. Methodology of MNPs modification.

Effect of experimental factors on sorption efficiency

The sorption of phytase onto coated magnetic sorbent depends on experimental parameters such as pH values of aqueous source, incubation time and temperature (Ozyilmaz *et al.*, 2005).

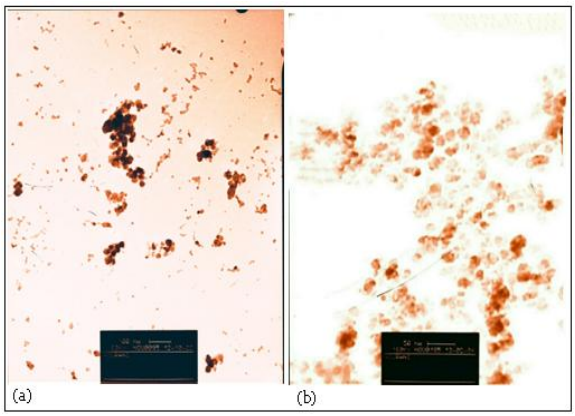

Fig. 2. TEM images of naked (a) and coated (b) MNPs.

The effect of pH (5-8) on phytase sorption efficiency by coated MNPs was investigated. The means of differences are shown in Figure 3. The sorbed enzyme in pH 7 and 7.35 was significantly greater than pH 5 ($P < 0.05$), but the differences with other treatments were not significant. This may reflect that the high acidity could decrease the sorption capacity of modified MNPs, significantly. The sorption of enzymes decreases with decreasing of the pH to 5, due to denaturation of protein related substances (phytase), and protonation of coated MNPs which change sorbent properties (Zhang *et al.*, 2013). Therefore, increasing of pH values (up to neutral pH) could increase the sorption efficiency.

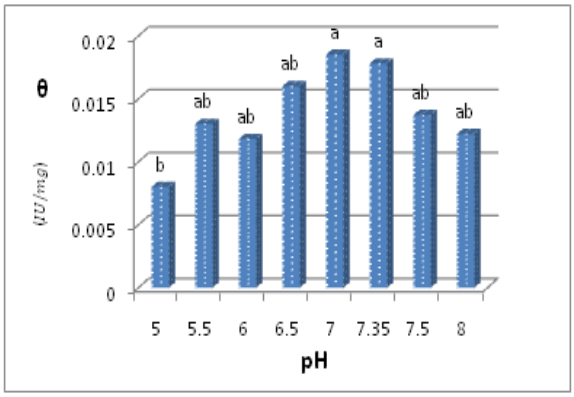

Fig. 3. The differences in phytase sorption (The means of each column with the same superscript are not statistically different).

The phytase sorption efficiency on modified MNPs was also considered. As shown in Figure 4, there are no significant differences among 30 and 10 minutes of shaking time, although 30 minutes treatment was numerically more than 10 minutes of shaking time. The adsorption efficiency was optimized with increase of shaking time. Therefore, the differences between 60 minute and other treatments were statistically significant ($P < 0.01$). Ren *et al.*, (2011) observed that by increasing the contact time up to two hours enzyme adsorption efficiency was optimized, which is in agreement with the present study.

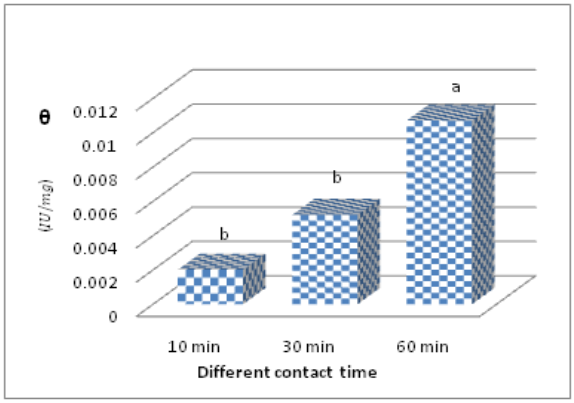

Fig. 4. Differences in phytase sorption capacity (The means of each column with the same superscript are not statistically different).

The adsorption of enzymes onto coated MNPs also depends on incubation temperature (Corrieia *et al.*, 2008; Shamim *et al.*, 2008). Desorption of phytase

were examined at different temperature. As shown in Figure 5, the most adsorption between different treatments was observed at 6 °C. The differences were statistically significant ($P < 0.01$). The most probable possibility for decreases of sorption efficiency with increasing of temperature is exothermic sorption of phytase on nano-sorben. In contrast to this, Shamim *et al.* (2008), obtained optimum enzyme adsorption in 25 °C and 40 °C.

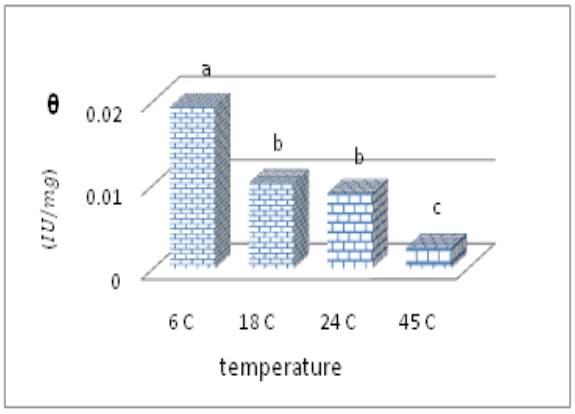

Fig. 5. Differences of phytase sorption (The means of each column with the same superscript are not statistically different).

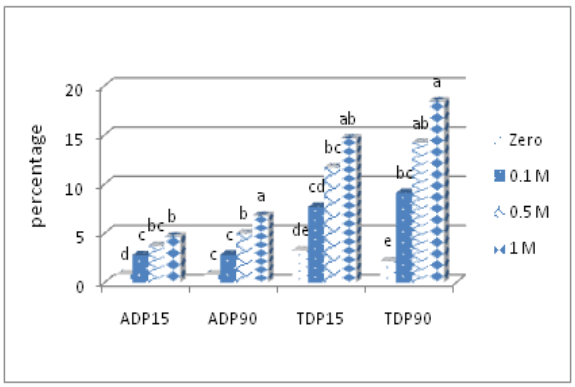

Fig. 6. ADP and TDP in different shaking times (15 and 90 minutes) and various concentrations of NaCl solution (zero, 0.1, 0.5, and 1).

Optimized parameters

The optimum result of pH values, mixing time and incubation temperatures were determined at pH 7, 60 minutes and 6 °C, respectively. These optimized conditions were used for desorption stage.

Desorption of phytase

Desorption of sorbed phytase was performed to evaluate the capacity of enzyme releasing and its activity. Desorbed phytase activity was determined in different treatments. Apparent and true desorption percentage are shown in Figure 6. The ADP differences of 1 M NaCl solution in 90 minutes of shaking time were statistically significant compared to other treatments ($P<0.01$).

The highest TDP (more than 18%) was recorded at 90 minute time in 1 M NaCl solution. The desorption behavior versus shaking time and NaCl concentration reflect that desorption percentage increases with increasing of shaking time and concentration of NaCl solution. These results are reasonable because of higher ionic strength in solution and more effective contact time for enzyme separation.

Conclusion

The morphology of coated MNP_s via cibacron blue polymerization was analyzed using TGA, TEM, and FT-IR Methods. Modification of MNP_s was confirmed by FT-IR and TGA results. The highest desorption efficiency (lower than 20%) was achieved at 1 M NaCl solution in 90 minutes of contact time. Various factors such as pH values, contact times, temperature of shaking, and concentration of NaCl solution had a significant effect on sorption and desorption of phytase. The obtained results show that cibacron blue grafted on MNP_s as a nano-sorbent could be a low cost, facile, and rapid device for phytase extraction.

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