Rational Design of Monodisperse Mesoporous Silica Nanoparticles for Phytase Immobilization

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ABSTRACT: Monodisperse mesoporous silica nanoparticles (MMSNs) with fractal structures were synthesized via a facile, one-pot, surfactant-free process under the well-known Stöber synthesis condition. It was characterized by scanning electron microscope, transmission electron microscopy, and N₂ adsorption–desorption isotherms. Phytase was immobilized on the MMSNs by physical adsorption. The enzyme loading capacity, activity, and release profile were measured by a faster and more reliable assay method, which was based on the hydrolysis of para-nitrophenylphosphate. The results show that the fractal structures have an important influence on the phytase capacity, and the releasing results also illustrated that phytase immobilized on MMSNs possessed the smallest releasing amounts under acidic conditions (pH = 3).

1. INTRODUCTION

Phytase is a class of phosphatases, which causes dephosphorylation of phytate. It is an exogenous phytase added in animal feed for improving the availability of phosphorus and minerals and for alleviating environmental problems caused by animal excretion. However, the maximum use of the phytase activity still has some challenges when used in supplementing feed. The pH in the small intestine is in the range of 5.7–7.0, and the pH in the gizzard is in the range of 2.5–4.7 for poultry. It is known that the enzyme is easy to deactivate under acidic environment. Hence, these limitations could decrease the phytase activity and availability.

Approaches to protect the activity of phytase in acidic gizzard conditions target its release, and prolong its activity at the small intestine include enzyme encapsulation in the polymer matrix and enzyme immobilization on an insoluble support. However, enzyme encapsulation in the polymer matrix always has a poor accessibility with the substrate. The immobilization technique provides greater resistance to denaturation when the enzyme is exposed to high temperatures and acidic pH media. Therefore, it remains a challenge to find a support that presents the specific characteristics for efficient phytase immobilization using a simple and rapid method. Besides, the support should be nontoxic and be able to be added to animal feed. Mesoporous siliceous materials possess controllable morphology and mesostructures, developed porosities, high level of biocompatibility, and ease of functionalization. These materials have been prospected as promising carriers for enzyme immobilization. Here, the phytase size is in the range of 4–10 nm, so the suitable mesoporous silicas are KIT-6, SBA-15, and SBA-16. There are very few literatures about phytase immobilized in mesoporous silica materials. Belamie has studied the phytase immobilized in KIT-6 with benefits to plant phosphorus nutrition, which gives us some instructions. The free phytase activity dropped rapidly at 55 °C, compared to immobilized phytase on KIT-6 under the same condition, which suggested that the thermal stability of phytase was greatly enhanced upon immobilization in the mesoporous silica. However, the activity of immobilized phytase over phytate is much smaller compared to free phytase owing to the diffusion constraints in the mesoporous materials. This disadvantage cannot meet the requirement in poultry feed industry. In addition, they compared the effect of pore size of the mesoporous materials on the enzyme loading amount. The pore diameter for MCM-41 is 3.8 nm and that of KIT-6 is 8.6 nm. The protein is bigger than the pore of MCM-41 but smaller than the pore of KIT-6. The specific loading amount of phytase is higher than that of MCM-41. Besides, Belamie et al. claimed that the phytase loading amount increased with the increase in phytase concentration. However, the effect of the pore channel structure on the loading amount and enzyme activity has not been studied.

Olsson et al. studied the effect of pore size, pore surface area, and pore volume of mesoporous silica materials on the feruloyl esterase loading amount and activity. Three kinds of mesoporous silica materials, for example, SBA-15, MCF, and KIT-6 were chosen to study the enzymatic performance of immobilization Fae to catalyze the reaction of MFA to BFA.
and FA. The results suggest that the pore size is a major factor limiting protein immobilization. As the pore size of SBA-15 was increased, more proteins were immobilized on it. Besides, geometry also plays a role in immobilization efficiency. The straight pore channel has a negative effect on immobilization, but the mechanism of protein diffusion into the pores of mesoporous silica of different geometries needs to be the subject for further investigation. Farha et al. studied the effect of crystal length on enzyme diffusion, who provided a method to study the enzyme diffusion behavior in the mesoporous materials. They synthesized two different lengths of crystals with arrayed channel-like pores and used an Alexa fluor 647-labeled enzyme and then examined the fluorescence intensity profile with time. The results demonstrated that larger MOFs need longer time for enzyme immobilization. Even PCN-600 had the maximum loading amount in the pore channel, for the accessibility of the enzyme was still very small. Hence, except for considering the enzyme-loading amount on the carrier, the enzyme accessibility is another important factor that needs to be considered.

Monodisperse mesoporous silica nanoparticles (MMSNs) possess a high stability and good biocompatibility. The pore size ranges from 4–10 nm. It has a fractal morphology, which is accessible from all sides compared to tubular pores of MCM-41 and SBA-15. This improved accessibility allows increased loading of active sites onto the silica surface without blocking the pores, more importantly, allows increased accessibility of these generated active sites. We assume this kind of structure has a good accessibility for enzyme contacting with the substrate. Besides, the rough surface is easily adhesive to the target site, which can improve the enzyme efficiency and reusability. SBA-15 has a hexagonal structure with round, straight pores of defined sizes, while KIT-6 has a 3-dimensional channel-like pore. In our work, three kinds of ordered mesoporous silicas (MMSNs, SBA-15, and KIT-6) with approximately similar pore sizes were successfully synthesized to investigate the effect of the pore channel structure on the activity of phytase. We measured the phytase loading amount, phytase release performance under different pH media, and the phytase activity of the immobilized enzyme on different mesoporous nanoparticles. This systematic study enabled elucidation of the relationship between the mesostructured geometry and phytase activity.

2. RESULTS AND DISCUSSION

White powders were obtained via a facile process without using a surfactant, which was a green and environmentally friendly approach. As we can see from the transmission electron microscopy (TEM) (Figure 1) and scanning electron microscopy (SEM) (Figure 2) images, spherical MMSNs (Figure 1a) are uniform with a diameter of 310 nm, which were well suspended without aggregation (Figure 2a). MMSNs possess a radially oriented structure with open pore channels, which is easily accessible to the internal surface. In addition, it is relatively easy to load a large amount of biomolecules in these sub-100 nm channels. SBA-15 (Figure 2c) has a rodlike morphology with a straight pore channel length of 1.2 μm (Figure 1c) that is further aggregated into approximately 5 μm long wheat-like structures. The KIT-6 samples also maintain the polyhedral particles and aggregate into larger entities (Figure 2b), which possess a tridimensional cubic network. Compared to MMSNs, both SBA-15 and KIT-6 have a microsized length pore channel. It is clear that the dispersity and pore channel of nanoscaled MMSNs are facile to accelerate the adsorption of phytase compared to KIT-6 and SBA-15. The short, open, and unobstructed channels in MMSNs lead to high phytase adsorption.

The N₂ sorption experiment was done to further elucidate the textual properties of the three kinds of samples. As we can see from the N₂ adsorption–desorption isotherms (Figure 3), type IV isotherms with a H₂-type hysteresis loop exist between the relative pressure ranged from 0.6–0.85 for KIT-6 (Figure 3c) and SBA-15 (Figure 3e), which is typical of mesoporous silica materials. Meanwhile, MMSNs also possess a type IV isotherm with an H₁-type hysteresis loop in the relative pressure range of 0.6–1.0 (Figure 3a), implying the presence of variously sized, slit-shaped mesopores. From the pore size distribution picture, we can find that all the nanoparticles have mesostructured pores. Among them, KIT-6 (Figure 3d) and SBA-15 (Figure 3f) have concentrated pore size distributions, while MMSNs (Figure 3b) have a broad pore size distribution. The Brunauer–Emmett–Teller (BET)
specific surface, pore size, and pore volume are summarized in Table 1. The pore size distribution of MMSNs ranges from 2.5 to 21 nm, and the most probable pore distribution is 9.7 nm. The pore mouths were about 21 nm from the N\textsubscript{2} adsorption isotherm. Another peak centered at 32.5 nm for MMSNs was attributed to the interparticle voids. Nevertheless, the pore size distributions of KIT-6 and SBA-15 are narrow, which is 9.2 and 7.6 nm, respectively. As expected, the longest axis of the phytase is slightly below 7.5 nm, which is smaller than the pores of all the mesoporous silica particles.

The specific loading experiments were performed using the following steps. The phytase solution was mixed with different initial concentrations of particle solution to study the effect of phytase concentration on the phytase loading amount. Phytase adsorption isotherms are presented in Figure 4, and it is clear that the specific loading capacity of SBA-15 and KIT-6 decreased with the increase of phytase concentration except for MMSNs.

As we can see the loading capacity from Table 2, at a phytase initial concentration of 0.5 mg/mL, the loading amounts of phytase on SBA-15 (335.1 \(\mu\)g/mg) were higher than that of

| sample  | BET specific area (cm\(^2\)/g) | pore size (nm)\(^a\) | pore volume (cm\(^3\)/g)\(^a\) |
|---------|-------------------------------|----------------------|-------------------------------|
| MMSNs   | 378.7                         | 8.2                  | 0.97                          |
| KIT-6   | 613.2                         | 9.2                  | 1.04                          |
| SBA-15  | 543.3                         | 7.6                  | 1.00                          |

\(^a\)Calculated from desorption branch by the BJH method.
MMSNs (214.1 μg/mg) and KIT-6 (229.2 μg/mg). When increasing the phytase initial concentration to 1 mg/mL, the phytase loading amounts on MMSNs were increasing to 237.1 μg/mg, which is similar to that of SBA-15 and higher than that of KIT-6 (136.9 μg/mg). If the phytase initial concentration was increased, the phytase loading amounts on all of the silica particles were decreasing. Among them, the down trend of phytase loading amounts on MMSNs was smaller than that of SBA-15 and KIT-6. As a result, with a lower initial phytase concentration, the specific loading amount on SBA-15 is larger than the other two particles, which is because of the large specific area of SBA-15 (Figure 4). When increasing the initial phytase concentration, the specific loading capacity of SBA-15 and KIT-6 decreased dramatically. As for the tubular channel of SBA-15 and KIT-6, phytase is easily clogged in the pore aperture. Nevertheless, the radical pore channel of MMSNs can avoid phytase clogging during the loading process. This suggests that the geometry also plays a role in immobilization efficiency. The straight pore might be more accommodating to the protein loading. It is known that protein is easy to accumulate when it encounters a bend, so the tortuous channel structures that exist in KIT-6 may be prone to protein clogging, while MMSNs have an improved accessibility to internal surface. When increasing the initial phytase concentration, the specific loading amounts change slightly.

In order to investigate the effect of mesopore channels on the phytase activity after loading on mesoporous silica particles, we test the phytase activity after loading on mesoporous silica particles. To gain the results, phytase activity was measured based on the hydrolysis of para-nitrophenylphosphate (p-NPP) to phosphate and para-nitrophenol. The rate of para-nitrophenol formation was determined by the change in absorbance at 410 nm. As we see from Figure 5, the phytase activity decreased after immobilization compared to free phytase under the same conditions. The phytase initial concentration also affected the phytase activity. The phytase activity immobilized on MMSNs, KIT-6, and SBA-15 was listed in Table 3. The specific activity of phytase in MMSNs increased with the increase of phytase initial concentration. For SBA-15, the maximum of phytase activity reached at 1 mg/mL phytase initial concentration.

While phytase initial concentration had little effect on the phytase activity immobilized on KIT-6, the specific activity of phytase immobilized on SBA-15 was larger than that of MMSNs and KIT-6 under the same initial concentration. However, the phytase activity immobilized on SBA-15 reaches the maximum with the 1 mg/mL phytase initial concentration. If the phytase initial concentration was increased, the phytase activity immobilized on SBA-15 decreased, which may be due to the clog in the entrance for a larger enzyme concentration. Phytase immobilized on KIT-6 possesses the smallest activity which may be attributed to the twisting of the pore channel and lower accessibility of the substrate.16

The physical adsorption of enzyme-support linkage only through electrostatic interaction is not enough to retain the protein within the siliceous materials. Therefore, under different pH or dilution conditions, phytase released from the particles is unavoidable. Releasing experiments from different particles were performed under different pH buffers. As we can see from Figure 6, these three mesoporous silica materials reached an equilibrium point after 1 day. The release percentage of MMSNs is 14% after 24 h under pH = 3 media condition, which is lower than that of SBA-15 (36%) and KIT-6 (44%). It indicates that phytase has a larger interaction with MMSNs because of electrostatic force. The results give us a good prediction that phytase immobilized on MMSNs remains as a large loading amount when passing through the acidic digesting tract. Hence, phytase immobilized on MMSNs can be used as supplementing feed in animal feed. The pH value of the small intestine is in the range of 5.7–7.0, so the releasing profiles of phytase under pH = 5.5 and pH = 7 conditions were also measured. The results illustrate that the phytase immobilized on MMSNs has a 44% releasing percentage under pH = 5.5 media, while phytase immobilized on SBA-15 and KIT-6 has 74 and 64%, respectively. For pH = 7 media, the releasing percentages of phytase immobilized on MMSNs, SBA-15, and KIT-6 are 34, 31, and 77%, respectively. On the one hand, phytase immobilized on MMSNs can target-release in the small intestine and the oriented radiation pore channel can increase the accessibility of the substrate with the phytase;
on the other hand, the rough surface of MMSNs also can help MMSNs adsorb onto the villi of the small intestine, which can prolong the activity of the phytase in the small intestine.

3. CONCLUSIONS

MMSNs with fractal structures were successfully synthesized via a facile, one-pot, surfactant-free method. Compared to bulk SBA-15 and KIT-6, the phytase enzyme can be efficiently immobilized onto nanosized MMSNs by means of a simple and fast adsorption protocol. The loading results showed that the fractal pore structure of MMSNs was beneficial for the adsorption of phytase enzyme compared to the long and straight pore channel. In addition, the releasing results also illustrated that phytase immobilized on MMSNs possessed the smallest releasing amounts under acidic conditions (pH = 3), which suggested that the enzyme would be as active as the soluble enzyme in the gastrointestinal conditions of monogastric animals. Besides, the phytase mainly release under the intestinal environment and the rough surface of MMSNs can adsorb onto the villi of the small intestine to prolong the activity of the phytase. The fractal structure of MMSNs has a great advantage to increase the accessibility to the substrate. Hence, it can provide long and efficient utilization of phytase added into the animal feed.

4. EXPERIMENTAL SECTION

4.1. Synthesis. MMSNs were synthesized via a facile, one-pot, surfactant-free process under the well-known Stüber synthesis condition. Typically, an aqueous-alcoholic solution was prepared by mixing ethanol (40 mL), distilled water (10 mL), ammonium hydroxide (1.56 mL), and ethylenediamine solution (0.225 mL) under stirring at 60 °C. After that, 3-aminophenol (0.412 g), formaldehyde solution (0.9 mL), and TEOS (1.56 mL) were added to the abovementioned solution and stirred for another 5 h. Then, the solution was transferred to a Teflon hydrothermal reactor at 100 °C for 24 h. The as-synthesized composites were collected by centrifugation, ethanol washing, and drying. Finally, monodisperse mesoporous silica nanoparticles were harvested after calcination in air, noted as MMSNs. Here, KIT-6 and SBA-15 were synthesized according to the previous reports.

4.2. Characterization. The physical porous structure of materials was characterized by N2 adsorption–desorption isotherms with an ASAP Tristar II 3020 apparatus from Micromeritics. Prior to the sorption analysis, all the materials were degassed at 150 °C for 12 h. The surface area was determined by the BET methods. The pore size was calculated by desorption branch of isotherms.

The morphology of the samples was observed using a JEOL JSM 7800 field emission electron microscope. TEM images were obtained with JEOL 1010 operated at 100 kV. For TEM measurements, the samples were prepared by dispersing the powder samples in ethanol, after which they were dispersed and dried on carbon film on a Cu grid.

4.3. Phytase Activity Assay. In our experiments, the phytase activity was measured based on the hydrolysis of p-NPP to phosphate and para-nitrophenol. The rate of para-nitrophenol formation was determined by the change in absorbance at 410 nm. The assay was carried out using 96-well microtiter plates, where each well contains 10 μL of 5 mM p-NPP substrate in 0.25 mol/L of acetate buffer containing Tween 20 (pH 5.5). The solution was incubated at 37 °C in a static incubator. 10 μL of the enzyme solution was added to the designated well, then 90 μL of the prewarmed 5 mM p-NPP substrate solution was added to each well, the timer was started on the first addition, and the substrate was added to the wells. Then, 90 μL of acetate buffer (pH 5.5) was added to the wells. The plate was covered with a plate seal and incubated at 37 °C in a static incubator for 15 min. At last, 100 μL of 1 M NaOH was added to each well in the same order as the substrate addition. The absorbance at 410 nm was recorded on the plate reader.

4.4. Phytase Immobilization on Mesoporous Silicas. Calculated amounts of silica materials were mixed with phytase (in acetate buffer solution at pH 5.5) with initial concentrations of 2, 1, and 0.5 mg/mL, respectively. The mixture was shaken for 20 h at room temperature. Then, the phytase–silica mixtures were centrifuged at 13,000 rpm for 15 min, and the residual concentration of phytase was measured. The specific loading amount of phytase was calculated according to the following equation

\[ L = \frac{(c_i - c_f)}{c_p} \]
Here, $L$ is the phytase loading efficiency; $c_i$ is the initial phytase concentration ($\mu g/mg$); $c_f$ is the phytase concentration in the supernatant after immobilization ($\mu g/mg$); $c_p$ is the silica particle concentration ($\mu g/mg$). The concentration of phytase was also recorded according to the phytase activity assay. We denoted the immobilized sample as MMSN/I, SBA-15/I, and KIT-6/I.

4.5. Phytase Release Experiments. First, phytase–silica particles with different initial concentrations were immobilized at room temperature. After enzyme immobilization, the mixtures were centrifuged, and the supernatant solution was removed. The acetate buffer solution was added with the same volume to redisperse the silica particles. The phytase concentration in the supernatant was recorded after 1, 2, 3, 4, 5, 10, 20, and 48 h. The concentration of phytase was also recorded according to the phytase activity assay.

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#### Notes
The authors declare no competing financial interest.

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