Biocatalysis and Agricultural Biotechnology 12 (2017) 96–103

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Improvement in biochemical characteristics of glycosylated phytase through immobilization on nanofibers

Javad Harati[a], Seyed Omid Ranaei Siadat[b,c,*], Hadi Taghavian[d], Saeed Kaboli[e,f], Shohreh Khorshidi[c]

[a] National Cell Bank of Iran, Pasteur Institute of Iran (IPI), Tehran, Iran
[b] Nanobiotechnology Engineering Laboratory, Faculty of Energy and New Technologies, Shahid Beheshti University, Tehran, Iran
[c] Protein Research Center, Shahid Beheshti University, Tehran, GC, Iran
[d] Department of Polymer Engineering, Islamic Azad University, South Tehran Branch, 1777613651 Tehran, Iran
[e] Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[f] Department of Biotechnology, Faculty of Biological Sciences and Technology, Shahid Beheshti University, Tehran, Iran

ARTICLE INFO

Keywords:
Phytase
Immobilization
Nanofiber
Starch and polyacrylamide
Response Surface Methodology (RSM)
Glycosylation
in silico

ABSTRACT

The objective of the current study was to enzymatically characterize immobilized phytase as a glycoprotein on an optimal blend of starch/PAAm nanofibers. *Aspergillus niger* phytase (phyA) was first expressed in *Hansenula polymorpha* using formate dehydrogenase promoter and α-mating factor signal peptide. Response surface methodology was then used to optimize the different levels of the enzyme, temperature and pH on the immobilized phytase. The electrospun nanofibers showed uniform morphology with an average diameter of approximately 269 nm. Afterward, the kinetic parameters of Km (56 μM) and Vmax (401 μmol/min mg) of the immobilized enzyme improved, but the optimum pH and temperature had not changed in comparison with the soluble phytase. The applicability of the nanofiber was confirmed by its outstanding immobilization efficiency (193%) and suitable retention of catalytic activity of the immobilized phytase (50.4% after the tenth repetition). Optimal orientation of the enzyme on the nanofiber using the *in silico* approach was predicted, which could improve the kinetic properties. The results demonstrate the good potential of *H. polymorpha* as an efficient expression system for the production of glycoproteins for immobilization.

1. Introduction

Phytase (myo-inositol hexaphosphate phosphohydrolase) is a class of phosphatases which catalyzes the stepwise hydrolysis of phytate to less-phosphorylated phytate and inorganic phosphates (Lei et al., 2013). Phytase has been used mainly for feed supplementation of simple-stomached animals, with the aim of improving availability of phosphorus, minerals, amino acids, and energy (Haefner et al., 2005) and alleviating environmental problems caused by excessive phosphorus in monogastric manure (Mullaney et al., 2000). Recent studies on phytase have demonstrated their application for improving human nutrition (Hurrell et al., 2003; Troesch et al., 2013) and synthesis of lower phosphoric esters of myo-inositol for their potential health value (Greiner et al., 2013; Quan et al., 2003). Chemical synthesis of myo-inositol phosphate isomers is difficult (Haefner et al., 2005), involves many steps and results in a low yield (Plettenburg et al., 2000). Because the enzymatic process presents high stereospecificity and mild reaction conditions, it is a useful alternative for this task.

Applications for immobilized enzymes in industry have benefits over soluble enzymes such as stability, volume-specific biocatalyst loading, recyclability and simplified downstream processing (Liese and Hilterhaus, 2013) however, conformational changes during immobilization may alter the catalytic efficiency of the enzymes (Secundo, 2013). It has been reported that immobilization can change the optimum pH (Doğaç and Teke, 2013; Wang et al., 2014) and the optimum temperature of the enzymes (Bahar, 2014; In et al., 2007; Kumar et al., 2014). It also has been reported that the enzyme concentration could affect immobilization yield (Zhang et al., 2011). In other studies, after immobilization, the values of Km and Vmax were increased and decreased, respectively (Babaei et al., 2014; Rehman et al., 2014). It can be deduced that enzyme immobilizations significantly affects the enzymatic characteristics.

Many researchers have reported on phytase immobilization using different strategies. Greiner et al. (Greiner et al., 2013) immobilized

* Corresponding author at: Nanobiotechnology Engineering Laboratory, Faculty of Energy and New Technologies, Shahid Beheshti University, Tehran, Iran.
E-mail addresses: Javad.harati6@gmail.com (J. Harati), o_ranaei@sbu.ac.ir, Rsiadat@gmail.com (S.O. Ranaei Siadat), Hadi.taghavian@gmail.com (H. Taghavian), Kaboli2009@gmail.com (S. Kaboli), shohre.khorshidi@gmail.com (S. Khorshidi).

http://dx.doi.org/10.1016/j.bcab.2017.08.009
Received 29 December 2016; Received in revised form 15 August 2017; Accepted 17 August 2017
Available online 18 August 2017

phytase on magnetic nanoparticles in order to produce partially myo-inositol phosphate. They showed that phytase immobilization decreased catalytic turnover considerably. Ushasree et al. (Ushasree et al., 2012) immobilized phytase on starch agar bed for the dephytination of soy milk. They showed a shift in optimum pH toward acidic pH. Phytase has also been immobilized as a part of a biosensor to measure phytic acid (Cruz Rodrigues et al., 2011) as well for use in the feed industry (Cho et al., 2011; Onem and Nadaroglu, 2014). Mesoporous silica, alginate/carrageenan beads and modulated alginate hydrogels have been used for phytase immobilization as well (Awad et al., 2015; Trouillefou et al., 2015; Zhang and Xu, 2015). Bioreactors are another goal of phytase immobilization to produce special breakdown products from phytate (Greiner and Konietzny, 1996).

By providing a wide range of advantages such as high porosity and interconnectivity along with an inexpensive processing method (Wang et al., 2009), nanofibers present a new prospect for phytase applications. In a recent study by the authors (Taghavian et al., 2015), it was demonstrated that electrospun-nanofibers obtained from the 2.7 g/ml solution of a blend of 35% (W/V) starch:polyacrylamide (PAAm) provided the best approach for the preparation of an active immobilized phytase formulation. In the present study, *Hansenula Polymorpha* is selected as a suitable host for producing recombinant glycoprotein (Gellissen and Kang, 2002), and the positive impact of glycosylation on the immobilization efficacy of phytase is considered (Bakker et al., 2000). Afterwards, the biochemical characteristics of phytase immobilized on an optimum blend of starch/PAAm nanofibers are surveyed. The results present an improvement in the kinetic properties of the immobilized phytase without changes its optimum temperature and pH in comparison with the soluble enzyme.

2. Materials and methods

2.1. Chemicals

Wheat starch was obtained from the domestic market. The 3-aminopropyltriethoxysilane (APTES) and phytic acid dodecasodium salt hydrate were purchased from Sigma (USA). Yeast nitrogen base (YNB) was bought from HiMedia (India). Glutaraldehyde (GA) was obtained from Panreac Quimica SA (Spain). Formic acid (~98%) was purchased from Merck (Germany). *Escherichia coli* (*E. coli*) *DH5α* was purchased from Sinaclone (Iran). Shuttle vector (pFPMTMFα) and *H. polymorpha* (URA3-) were bought from Artes (Germany) and were used as expression plasmid and expression system, respectively. All other chemicals and reagents were of analytical grade and purchased from Merck (Germany).

2.2. Cloning, expression and purification of phytase

The 3-phytase gene (phyA) from *Aspergillus niger* with 444 amino acid residues was designed and synthesized based on the codon usage frequency of *H. polymorpha*. Cloning and cell transformations in *E. coli DH5α* were performed according to Sambrook et al. (1989)). All procedures of genetic manipulation of *H. polymorpha* were performed according to the Artes protocol. Briefly, pFPMTMFα containing the PhyA gene was transformed to *H. polymorpha* through electroporation and screened based on the auxotrophic marker (URA3-). After selection, transformants were grown for approximately 30–80 generations under selective conditions to increase the plasmid copy number and incorporate the plasmids into the genomic DNA of the host.

Yeast cells were grown overnight in YPD media containing 1% yeast extract, 2% peptone and 2% glucose as a preculture at 37 °C. Then it was centrifuged for 8 min at 2000 rpm to pellet the cells to inoculate the expression medium. Expression was done in a 250 ml shaker flask containing 25 ml of YNB media at 37 °C that was shaken at 250 rpm. The YNB media contained 0.17% YNB, 1% glycerol and 0.5% ammonium sulfate in distillated water and the pH was adjusted to 6.0. Samples (1 ml) were taken at 24-h intervals for four days to analyze the biochemical activity. The extracellular activity was determined in the cell-free supernatant. Purification was done using a Superdex 200 10/300 GL (GE Healthcare). The gel filtration column was equilibrated with 200 mM Na-acetate buffer (pH 5.5). The FPLC was run at 25 °C and a flow rate of 0.9 ml/min. After eluting the unbound untargeted proteins, the fractions containing active recombinant phytase were pooled and further analysis was done by SDS-PAGE.

2.3. Protein determination

Protein concentration was measured by using the Bradford protein assay (Bio-Rad Laboratories; USA) against the standard curve of bovine serum albumin (Bradford, 1976).

2.4. Enzyme activity assay

Phytase activity was determined by measuring the amount of inorganic phosphate liberated from the sodium phytate using the ammonium molybdate method (Heinonen and Lahti, 1981) with slight modifications. The assay mixture consisted of 0.5 ml of 2.5 mM sodium-phytate (in 0.1 M pH 5.0 Na-acetate buffer) and 0.475 ml of 0.1 M pH 5.0 Na-acetate buffer. The enzymatic reaction was started by adding 25 µl of soluble enzyme to the assay mixture (different amounts of phytase were applied for immobilization; Table 1). After incubation for 30 min at 50 °C, the reaction was stopped by adding 2 ml of freshly prepared color reagent solution (25% ammonium molybdate solution + 25% sulfuric acid 5 N + 50% acetone). After 45 s for color development, 0.1 ml of 1.0 M citric acid was added to each test tube and the absorbance was read at 380 nm. To avoid external mass transfer limitations during the immobilized enzyme assays, the reaction media was slightly shaken every 5 min. One unit (1 U) of phytase activity was defined as the amount of enzyme that released 1 µmol inorganic phosphate per minute under the assay conditions.

2.5. Polyacrylamide synthesis, nanofiber production, cross-linking and enzyme immobilization

All procedures of nanofiber synthesis and immobilization are shown in Fig. 1. PAAm was prepared as described previously (Taghavian et al., 2015). The resulting PAAm was precipitated with acetone and kept in a vacuum oven for 8 h at 60 °C. Based on the results of the previous study by the authors (Taghavian et al., 2015) using various percentages of starch and PAAm, a blend of 35% (W/V) starch with 2.7 g/ml PAAm was selected and dissolved individually in formic acid at 50 °C for 2 h. Next, they were blended and stirred at room temperature for 12–14 h to obtain the finest homogeneous solution which showed no phase separation prior to spinning after proper formation of the solution.

Electrospinning was performed using a 1 ml syringe at an electrical potential difference of 15 kV and a distance of 20 from the needle to the collector. The pump (SP-500 model) was set to deliver the solution at a flow rate of 0.1 ml/h. The collector was a flat support covered with aluminum foil. The nanofibers obtained were dried in an oven at 60 °C for at least 10 h to remove the remaining solvent before use. Crosslinking of the nanofibers was done following treatment with APTES (20% (V/V) in methanol at 50 °C for 4 h) and GA (1% (V/V) in

Table 1
Coded value of independent variables.

Coded value	Factor	Unit	Level				
			-2	-1	0	+1	+2
A	Temperature	(°C)	30.00	38.11	50.00	61.89	70.00
B	pH	–	3.00	4.01	5.50	6.99	8.00
C	Enzyme Unit	Unit/ml	0.50	1.01	1.75	2.49	3.00