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Purification and biochemical characterization of an *Aspergillus niger* phytase produced by solid-state fermentation using triticale residues as substrate

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ABSTRACT

In this study, an extracellular phytase produced by *Aspergillus niger* 7A-1, was biochemically characterized for possible industrial application. The enzyme was purified from a crude extract obtained by solid-state fermentation (SSF) of triticale waste. The extract was obtained by microfiltration, ultrafiltration (300, 100 and 30 kDa) and DEAE-Sepharose column chromatography. The molecular weight of the purified enzyme was estimated to be 89 kDa by SDS-PAGE. The purified enzyme was most active at pH 5.3 and 56 °C, and retained 50% activity over a wide pH range of 4 to 7. The enzymatic thermostability assay showed that the enzyme retained more than 70% activity at 80 °C for 60 s, 40% activity for 120 s and 9% after 300 s. The phytase showed broad substrate specificity, a K_m value of 220 µM and V_{max} of 25 µM/min. The purified phytase retained 50% of its activity with phosphorylated compounds such as phenyl phosphate, 1-Naphthyl phosphate, 2-Naphthyl phosphate, p-Nitrophenyl phosphate and Glycerol-2-phosphate. The inhibition of phytase activity by metal ions was observed to be drastically inhibited (50%) by Ca^{++} and was slightly inhibited (10%) by Ni^{++}, K^{+}, and Na^{+}, at 10 and 20 mM concentrations. A positive effect was obtained with Mg^{++}, Mn^{++}, Cu^{++}, Cd^{++} and Ba^{++} at 25 and 35% with stimulatory effect on the phytase activity.

1. Introduction

Phytases are hydrolytic enzymes (phosphatases), belonging to the subfamily of histidine acid phosphatases. They catalyze the hydrolysis of phytate phosphomonoester bonds (salts myo-inositol hexakisphosphate) or myo-inositol 1, 2, 3, 4, 5, 6- hexakis dihydrogenphosphate (phytic acid), and produce derivatives such as tetra, tri, di and inositol monophosphate, as well as inorganic phosphate (Pi) [1,2]. The main application of this enzyme is in the animal feed industry, where it is used as feed supplement for non-ruminant animals (such as pigs, chickens, turkeys, etc). This is because phytic acid is the largest reservoir of phosphorus in plants: 60–80% of plant phosphorus is contained in phytic acid. Since their digestive system lacks phytase, monogastric animals are incapable of metabolizing phosphorus. Phytic acid is excreted in their stool, causing soil pollution and eutrophication of water by phosphates [3–5].

During animal digestion, phytase liberates the Pi present in phytic acid [5]. When it is used as a supplement, Pi is reduced in manure by about 33%, which ensures a decrease in environmental pollution by a third, in addition to improved animal nutrition. The main limitation to the use of this enzyme with high nutritional and environmental interest, is the high market price and in some cases, low production levels and thermostability [6,7].

Phytase producing microorganisms include filamentous fungi of the genus *Aspergillus*. In various studies, these fungi were found to produce the most active extracellular enzyme with the most suitable characteristics of both pH and temperature stability. Hence, microorganisms of the genus *Aspergillus,* are the most used in the industrial production of this enzyme [8,6]. Solid-state fermentation is a process commonly applied for the production of extracellular enzymes [9].

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As a result of the favourable characteristics of phytase, as well as its practical application as an additive in the diets of non-ruminants, this enzyme has taken a position of great interest in biotechnological applications to reduce the phytate content of fodder and commercial foods [10]. Phytases used as additive must be effective in releasing phosphate from phytic acid and must demonstrate their effectiveness at the digestive tract level, as well as withstand the conditions of pH and temperature [11,12].

This paper reports the purification, characterization and enzymatic properties of a novel phytase produced by *Aspergillus niger* 7A-1, by solid state fermentation of the agro-industrial waste of triticale with special interest in its application in animal feed at the industrial level.

2. Materials and methods

2.1. Microorganism production and enzyme recovery

The *A. niger* 7A-1 strain was provided by the Nanobioscience Group, University Autonomy of Coahuila, Saltillo, México. Prior to the commencement of the study, the strain was grown and maintained on potato dextrose agar (PDA) slants at 28 ± 1 °C, to obtain the inoculum. After seven days of fungal growth, spores were collected with 0.1% Tween 80 solution. Their concentration was adjusted to1×10^6 spores/mL.

The substrate used for phytase production by solid state fermentation (SSF), was provided by the Agrarian Autonomy Universidad "Antonio Narro", México. It consisted of a mixture of the agroindustrial waste of triticale. The substrate was washed with distilled water to remove soil and impurities, dried at 60 °C, ground to obtain a particle size of approximately 0.3 mm, and stored in sealed bags until use.

The substrate (5 g) was placed in a Petri dish and sterilized at 121 °C for 20 min. After cooling, the substrate was moistened with 3 mL of sterile solution which contained: NH_4NO_3 (40 g/L), dextrose (168 g/L), lactose (4.8 g/L), Tween 80 (10 mL/L) and KCl (2 g/L) to adjust moisture at 60% approximately. The substrate was inoculated with 0.5 mL spore suspension (1×10^6 spores/mL) and the content was mixed and incubated for five days at 28 ± 1 °C under static condition.

The enzyme extract was obtained from the fermented samples with distilled water (5 mL/(g of substrate)) after stirring for 1 h (at 200 rpm and 25 ± 1 °C). The suspension was centrifuged at 10,000*g* for 10 min. The clear brown coloured supernatant was termed crude extract (CE) and stored at 4 °C until further use.

2.2. Phytase enzyme purification

The crude extract obtained by the SSF was concentrated using a Millipore Amicon ultrafiltration cell model 8200 (Bedford, MA, USA) with a 30, 100 and 300 kDa molecular cut-off PM30 Amicon membrane at 4 °C. The retained fraction was cleared by centrifugation (10,000*g* for 15 min at 4 °C). Subsequently, the pH of this concentrated extract was adjusted to 7.0 with 0.1 M NaOH solution. Thereafter, a 2 mL aliquot of the enzyme was loaded onto a 4.7 mL DEAE-Sepharose CL 6 B column (Pharmacia), pre-equilibrated with 50 mM sodium acetate at pH 5.15. The total unbound protein was removed by washing with two bed volumes of equilibration buffer. Bound protein was then eluted using a linear salt gradient (0 to 0.5 M NaCl in 50 mM sodium acetate buffer at pH 5.15 containing 0.5% (w/v) sucrose) with flow rate: 0.2 mL/min, 2.0 mL fractions were recovered. The fraction (2 mL) with the greatest specific activity was selected for subsequent analysis and characterization.

2.3. Enzyme activity assay

Phytase activity was determined by measuring the Pi released from sodium phytate solution [13,12]. The reaction mixture consisted of 1 mL of 0.1 M $MgSO_4$*$7H_2O$, 2.4 mL of 6.82 mM phytic acid and 0.6 mL of appropriately diluted crude enzyme solution. Solutions of $MgSO_4$*$7H_2O$ and phytic acid were prepared with 0.2 M sodium acetate buffer (pH 5.15). Subsequently, the reactants were incubated at 55 °C for 60 min, and the reaction was stopped by adding 0.5 mL of 10% trichloroacetic acid. Thereafter, 1 mL of distilled water and 2.4 mL of Taussky-Schorr reagent (10 mL of 10N H_2SO_4, 1 g of $(NH_4)Mo_7O_{24}$*$4H_2O$ and 5 g of $FeSO_4$*$7H_2O$ graduated to 100 mL distilled water) were added to generate a blue chromophore [13]. The content was mixed for 30 min and then the absorbance was determined at 660 nm. Measured values were correlated with a standard curve that was constructed using monopotassium phosphate. One unit of phytase activity was defined as the amount of enzyme that released 1 μmol of phosphate per minute under assay conditions. All the enzyme activity analyses were performed in triplicate.

2.4. Other analytical determinations

Protein concentration was determined by the Bradford method using bovine serum albumin as the standard at 0 to 20 μg/mL [14].

The K_m and V_{max} values were determined by means of enzyme activity assay performed using different phytic acid concentrations to plot the obtained results in Lineweaver-Burk coordinates [15].

2.5. Molecular characterization of A. niger purified phytase

One-dimensional SDS gel electrophoresis was performed using 10% (w/v) acrylamide gel in a vertical electrophoresis system, and staining was carried out using Coomassie Blue R-250 [16]. The molecular weight was determined using high molecular weight markers (Sigma-Aldrich Co.). For zymogram analysis, non-denaturing electrophoresis was carried out in the same manner, but with the omission of SDS from the gel running and loading buffers, the sample was not pre-treated under denaturing conditions. After electrophoresis, the gel was equilibrated with 0.2 M sodium acetate buffer at pH 5.15 for 30 min, and then incubated in the same buffer containing 0.04% (w/v) *p*-nitrophenyl phosphate (*p*NPP) at 55 °C for 30 min. After incubation, the gel was rinsed with distilled water, and then a 0.5 M Na_2CO_3 solution was added for visualization of yellow zones, indicating substrate hydrolysis [12].

2.6. Effect of temperature and pH on the purified enzyme activity

Enzyme thermostability. Phytase activity versus reaction temperature and pH were determined according to the method of Howson and Davis. [17]. A temperature range of 20–80 °C and a pH range of 2.0–8.0 were assayed. The following buffers were used: 0.2 M glycine-HCl (pH 2–3), 0.2 M sodium acetate (pH 4.0–5.5), 0.2 M 2-(N-morpholino) ethanesulfonic acid MES (pH 6.0–6.5) and 0.2 M Tris-HCl (pH 7.0–8.0). Thermostability was assayed using purified phytase preincubated at 80 °C for 300 s before enzyme activity determination by the previously described method [8].

2.7. Determination of substrate specificity

The specificity of the purified phytase for different substrates was evaluated by replacing phytic acid in the reaction mixture with other phosphate containing compounds. All substrates (listed below) were used at a concentration of 3 mM. The phytase activity was evaluated in accordance with the phytase activity assay [18].

2.8. Effect of metal ions on A. niger phytase activity

The inhibitory or stimulatory effects of metal ions on the phytase activity were determined. Before enzymatic activity assay, the enzyme was preincubated for 10 min at 39 °C in the presence of the different salts defined below, and applied at two different concentrations (10 and