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Phytase activity from *Aspergillus oryzae* AK9 cultivated on solid state soybean meal medium

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Abstract

Phytase from a soy sauce koji mould was produced by solid state fermentation using soybean meal as the main substrate. The cultivation was carried out at 30 °C for 96 h. Characterization of the crude phytase showed that the maximum activity was obtained when the reaction was carried out at 75 °C and pH 5.0. However, after 2-h storage at different pH, maximum remaining activities were obtained when stored at pH 3.6 and 5.5 possibly indicting the presence of two distinct phytase enzymes. However, at different temperatures, there was only one peak of maximum remaining activity after 2-h storage at 40 °C. The presence of trypsin and taurochlorate at 1.0% (w/v) did not affect enzyme activity. Digestion of pre-mixed chicken feed with the enzyme koji at approximately 500 U/kg feed in the presence of 1% each of trypsin and taurochlorate increased the phosphate and protein available for animal consumption.

Keywords: Animal feed enzymes; Soy sauce koji mould; Soy sauce koji enzymes; Solid substrate fermentation

1. Introduction

Phytase initiates the release of phosphorus from phytate (*myo*-inositol hexakisphosphate), the storage form of phosphorus present in various seeds and grains commonly used as raw materials for animal feed production. Phytase has been used as feed supplement to improve phosphorus nutrition and reduce phosphorus in excretory products of animal [1]. Furthermore, hydrolysis of phytate also prevents protein–phytate complex formation, leaving more free protein available to be digested and adsorbed for animal growth [1]. Most of the studies on phytase production have been carried out in fungi, and especially those in the genus *Aspergillus* sp. due to their high production yields and their low-pH tolerance [2]. In this study, phytase production was studied in a solid substrate fermentation with a fungal strain (*Aspergillus oryzae* AK9) normally used to produce soy sauce. For soy sauce koji preparation, AK9 is utilized for its protein digesting ability with cooked soybean. It was reasoned that this fungus would also exhibit phytase activity since phytate is a normal constituent of soybean [3] and would be safe for inclusion in animal fed since it is considered to be a food grade microorganism. Phytase in AK9 koji was characterized and the koji tested for its potential use as an animal feed supplement.

2. Materials and methods

2.1. Cultivation

A stock culture of *A. oryzae* AK9 (AK9) was grown in potato dextrose agar (PDA) at 30 °C for 4 days and stored at 4 °C until used. Koji inoculum was prepared by suspending the spores from a PDA culture in 5 ml of 0.1% Tween-80 solution. This suspension was used to inoculate koji medium (KJM) containing 10 g of soybean meal, 3 g of rice flour mixed with 6.5 ml of distilled water. Cultivation was carried out at 30 °C for 96 h.

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2.2. Crude enzyme extraction

AK9 koji prepared as above was resuspended in 20 ml of 0.1 M acetate buffer (pH 5.6) and left standing for 30 min with occasional shaking prior to filtration through cheesecloth with squeezing. The filtrate obtained was centrifuged at 5000 × *g* for 10 min to eliminate any particulate material and the supernatant was employed as an enzyme extract.

2.3. Phytase assay

A quantitative assay for phosphate released by phytase was done following [4]. Briefly, the reaction mixture contained 2 ml of enzyme extract and 4 ml of substrate solution composed of 0.84 g sodium phytate in 90 ml acetate buffer (pH 5.5) [0.18 g acetic acid (100%), 3.0 g sodium acetate·$3H_2O$, 0.15 g calcium chloride·$2H_2O$, 90 ml distilled water, adjusted to pH 5.5 with acetic acid and diluted to 100 ml]. The reaction was carried out at 37 °C for 65 min. The reaction was stopped by adding 4 ml freshly prepared colour reagent. The colour reagent was prepared by mixing 25 ml ammonium molybdate solution [10 g ammonium molybdate·$4H_2O$, 90 ml distilled water, 1 ml NH_3 (25%), adjusted to 100 ml with distilled water] with 25 ml ammonium vanadate solution [0.235 g ammonium vanadate added to 40 ml distilled water at 60 °C followed by slow addition of 2 ml nitric acid and adjustment to 100 ml with distilled water] followed by slow stirring, addition of 16.5 ml nitric acid (65%), cooling to room temperature and volume adjustment to 100 ml with distilled water. The colour developed was measured at 415 nm. The activity was calculated from OA_{415} of the reaction under standard conditions after subtraction with OA_{415} of the reaction mixture with the addition of stop mix prior to that of the enzyme. This will also subtract any other phytate hydrolyzing activity and phosphate present prior to the AK9 phytase reaction. One unit of phytase activity was defined as the amount of enzyme that released 1 μmol of inorganic phosphate in 1 min.

2.4. Other assays

Free phosphate content was determined by the addition of 4 ml of Engelen colour reagent (see earlier) to 5 ml of sample. The protein content was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, USA).

Table 1
Phytase production by *A. oryzae* AK9 on KJM media at various time intervals

Incubation period (h)	Phytase activity (U/g)
0	0.0
24	0.0
48	4.5
72	8.8
96	15.8
120	16.0

3. Results and discussion

3.1. Phytase production by A. oryzae AK9 in solid state fermentation

KJM was originally formulated for AK9 spore production. However, phytase production can be induced by various cereal grains, bran, seeds or seed meals [5–8]. Therefore, the major soybean meal component of KJM was replaced with other high phytate containing raw materials such as wheat bran and rice bran. In addition, the content of rice flour was also varied. Results showed that AK9 cultivated on KJM gave the highest phytase activity (Tables 1 and 2). The optimum incubation time was 96 h and yielded an activity of 15 U/g of koji. This was 1.8 times higher than the activity obtained at 72 h (8.8 U/g) and only slightly less than that obtained at 120 h (16.0 U/g). Therefore, crude extract from 96-h culture was used to determine the general properties of the enzyme.

3.2. Effect of pH on phytase activity and stability

The optimum pH for AK9 phytase activity was determined in the acidic range as the enzyme was aimed to be used as feed supplement. The buffers used were 0.1 M glycine buffer (pH 2.0–3.6), 0.1 M acetate buffer (pH 3.6–6.0) and 0.1 M Tris–HCl buffer (pH 6.0–7.0). Changes in buffer were necessary due to limitations in buffering

Table 2
Phytase production by *A. oryzae* AK9 on various solid media at 96-h cultivation

Amount of rice flour added to the medium[a] (g)	Phytase activity (U/g) from medium with different major ingredient		
	Soybean meal	Wheat bran	Rice bran
0	13.5	1.8	UD[b]
1	14.1	1.7	UD[b]
2	14.6	4.5	1.5
3	15.8[c]	4.2	1.8
4	10.6	3.9	1.7
5	7.7	3.7	1.5

[a] The medium contain 10 g of major ingredient and 6.5 ml distilled water.
[b] UD: undetectable level, i.e. less than 0.15 U/ml of phytase activity.
[c] The activity obtained from cultivation on KJM medium.