Production and characterisation of microbial phytases

Theses of Dissertation

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1 Introduction and objectives

Among biogenous elements probably phosphorous is found in the 5th highest level following carbon, hydrogen, oxygen and nitrogen. Phosphorous and sulphur are essential in the energy metabolism and the ossification of both animals and humans. Phosphorous is basic compound in nucleic acids, ATP and phospholipids. Moreover, it plays an important role also in the growth and has effects on the health status of bone as well as on the quality of the egg of poultry. For animals phosphorus-containing water and plants are the main sources of phosphorus in their diet. In the plant kingdom, phytic acid is a storage form of phosphorus, which is found in different amount in vegetable raw materials. Cereal grains, legumes and oil seeds contains huge amount of phytic acid. The feedstuffs originating from plant contain about 60-90% of their total phosphorus content in the form of phytate. Phytic acid and its salts are anti-nutritional factors, because it has the ability to chelate various essential divalent metal ions blocking or slowing down their absorption and utilization. Additionally, it is also able to form complexes with dietary proteins, starch and lipids as well as to inhibit a number of nutritionally important enzymes. Organic phosphorous may pass through the digestive tract of non-ruminant animals undigested (poultry, pig) – same is the situation in the human digestion tract – because they lack the intestinal phytase. Therefore, in intensive livestock farming the feed must be supplemented with inorganic phosphate, and the phosphorus bound in phytate is excreted in the manure. This is one of the main sources of numerous environmental problems. What is more, the increased emission of phosphorous often contributes to eutrophication of surface waters.

To decrease the phytic acid content and to release organic phosphorous in feed or foodstuff the hydrolytic activity of phytase enzyme is the most appropriate.

Nowadays phytase has been widely applied as feed additive in livestock farming, because it can improve the availability of organic phosphorus and reduce the amount of phosphorus released into the environment. Many companies producing industrial enzymes have phytase enzyme preparation for supplementation of feed. This enzyme may also have an importance in human nutrition, and should help to decrease the risk of diseases in certain groups of people such as vegetarians or in the population of developing countries who usually consume high amount of plant-based foods.

Recently, it has been recognised that the sequential hydrolysis of phytate results different myo-inositol phosphate intermediates that have health care and pharmaceutical effects. These intermediates reduce the risk of colon cancer, the level of serum cholesterol and

triglycerides in test animals, and reduce lipid peroxidation, and act as antioxidants. Production of special myo-inositol phosphate intermediates can be achieved only by enzymatic technology. Further advantages of specific enzymatic technology are that it decreases environmental pollution at mild conditions.

Phytase enzyme occurs widespread in the world, which results in the production of different source of enzyme. The catalytic mechanisms, optimal parameters, substrate specificities and stability of the enzyme are different depending on the producing microorganisms, and knowledge of these is the criteria of the industrial application. Usually enzymes originated from thermophilic organisms has been much more heat tolerant than enzymes from mesophilic or plant sources. Phytases from filamentous fungi are glycoproteins and catalyse the hydrolysis of phytic acid in different way. Properties of individual enzymes are varied from species to species even from strain to strain.

Thus the main goal of my research work is the production and characterization of phytase enzymes from different origin. For this purpose *Aspergillus* strains and thermophilic *Thermomyces lanuginosus* filamentous fungi have been chosen.

Main objectives

1. Screening the strains of *Thermomyces lanuginosus* and *Aspergillus* filamentous fungi for phytase activity
2. Development of enzyme fermentation
    2.1 Elaboration of inoculation method
    2.2 Optimisation of the composition of fermentation medium
        2.2.1. Quality and quantity of carbon and nitrogen source
        2.2.2. Effect of surfactant on enzyme activity
3. Purification of enzymes to homogeneity
4. Characterization of purified phytases
    4.1 Molecular weight
    4.2 Optimum parameters
    4.3 Effect of metal ions on enzyme activity
    4.4 Determination of kinetic properties

2 Materials and methods

Production of phytase was achieved by *Aspergillus* and *Thermomyces lanuginosus* filamentous fungi originating from different culture collections.

Submerged fermentations were carried out in shaken flasks on different substrates containing phytic acid, at temperature proper for the individuals strain (*A. niger* 28°C, *T. lanuginosus* 47°C) at 220 rpm for 4-7 days. The fermentation process was followed by daily sampling and measuring the enzyme activity and pH. Phytase activity was determined using phytic acid as substrate and the inorganic phosphate liberated was quantified. Ammonium molibdate in stop solution form yellow complex with phosphate. The amount of it can be determined by spectrophotometry on 415 nm. One unit was defined as the amount of enzyme that releases 1 μmol of inorganic phosphate from Na-phytate substrate per min under the assay conditions.

Central composite design was applied for optimisation experiments. The purification procedures were performed as a combination of different chromatographic steps, applying Fast Performance Liquid Chromatography system (FPLC, Pharmacia, Uppsala, Sweden) at 4 °C.

The Bradford method and light absorption at 280 nm were applied to determine protein concentrations. The molecular weight of the enzymes was detected by SDS polyacrylamide gel electrophoresis.

3 Results

In my research fourteen *Thermomyces lanuginosus* and eleven *Aspergillus* strains were screened for their ability to produce extracellular phytase enzyme. The strain with the best activity was selected for the elaboration of enzyme fermentation technology. After the enzymes were purified to homogeneity, the characterizations were performed.

Production and characterization of phytase from Thermomyces lanuginosus

Thermomyces lanuginosus strains were screened for their ability to produce extracellular phytase, and *T. lanuginosus* IMI 096218 proved to be the best one. The enzyme production was enhanced by optimisation of the culture medium. The enzyme activity increased when the medium was prepared with TRIS-maleate/NaOH buffer (pH=7.5). Effects of different natural substrates containing phytate such as soy flour, wheat bran, corn flour, rice flour on phytase production were investigated, and the highest activity were achieved on rice flour. The

phytase activity was 30 times higher in submerged fermentation on rice flour than on wheat bran, which is widely applied in the industry. The optimal rice flour concentration was 5 (w/v)%. The effect of different agitation speed on enzyme production was investigated. It can be observed that using 220 rpm agitation speed the enzyme production was faster, the maximal activity was achieved on the 4th day of fermentation. Applying 120 rpm the enzyme production was maximal on the 7th day of fermentation.

The effect of the fermentation medium supplementation with various additives such as Tween 80, citric acid and yeast extract in 0.1% concentration both in themselves and in all possible combinations on the production of enzyme was investigated. Citric acid clearly inhibits the production of phytase enzyme, nearly by 90%. The presence of Tween 80 surfactant enhanced the extracellular phytase activity the most. The effects of other members of the Tween series (Tween 20, Tween 40, Tween 60, Tween 65, Tween 80 and Tween 85) on enzyme secretion were investigated in concentration of 0.1 % and Tween 20 and Tween 40 proved to be the best ones. The effect of different concentrations of Tween 20 and Tween 40 were also examined, and the best results were obtained in the case of the addition of 0.1% Tween 40 surfactant to the medium. By optimisating the inoculation technique, the highest phytase activity was achieved after initiation with 5% of 40-hour inoculum. The enzyme yield maximised on the 2nd day of the fermentation. As a result of optimization process more than 50-fold increase in the enzyme activity was achieved (1400-2000U/l).

Summarising the results it can be established that on the basis of the optimisation experiments the following medium composition is recommended for phytase production by *T. lanuginosus* IMI 096218 strain: rice flour 50 g/l, $MgSO_4*7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4$ 0.1 g/l, $NaNO_3$ 8.6 g/l, Tween-40 1 g/l, in TRIS-maleate/NaOH buffer (pH=7.5).

The extracellular phytase from *T. lanuginosus* IMI 096218 strain growing under the optimized fermentation conditions was purified by a combination of chromatographic procedures after precipitation with ammonium sulphate. The phytase was purified about 9.1 fold with yield of 5.1 %. The purified enzyme was checked by polyacrylamide gel electrophoresis. Two protein bands with a molecular weight of approximately 60 kDa and 90 kDa were detected on SDS-PAGE.

Two optimal pH values were determined: pH=5.5 and pH=7.5. The maximal activity was measured at 70 °C. Kinetic parameters of phytase originating from *T. lanuginosus* IMI 096218 strain on sodium-phytate substrate were determined by linear Lineweaver-Burk plot: K_M=0.285 mM, v_{max}=0.126 mM/min, and Hanes-Woolf plot: K_M=0.312 mM, v_{max}=0.132

mM/min. The half-life time of enzyme at temperature 54-58 °C and pH range 5.0-7.5 is more than 100 min.

Effects of various metal ions on enzyme activity were investigated. The presence of 1 mM Fe^{2+}, Fe^{3+} and 5 mM K^{+}, Ca^{2+} and Mg^{2+} ions resulted in 13-22% increase of activity, whereas the presence of 5 mM Cu^{2+}, Zn^{2+}, Ag^{+} and Co^{2+} ions strongly inhibited the enzyme reaction. In these cases, the residual activities were 34-55%.

Production and characterization of phytase from Aspergillus niger

Among the tested mesophilic *Aspergillus* strains the highest phytase activity was achieved by *Aspergillus niger* F00735. Different natural substrates – rice flour, pea flour, corn flour, corn starch, corn grit, soy flour, wheat flour and wheat grit – were examined in respect of phytase productivity. Rice flour proved to be the best substrate in submerged fermentation. The effect of different inorganic – sodium nitrate, ammonium acetate, ammonium sulphate – and organic (urea) nitrogen sources were investigated on phytase production. Sodium nitrate proved to be the best one.

The Central Composite Design (Response Surface Method) was used to determine the optimal concentration of the sodium nitrate and rice flour. On the 6^{th} day of fermentation the following model was achieved:

$$z=-846-634x+50x^2+7712y-5510y^2+273xy$$

where z: phytase activity [U/l]

x: concentration of rice flour [(w/v)%]

y: concentration of sodium-nitrate [(w/v)%].

The two factor- and their combined effects have an impact on the enzyme production at least 95% confidence level. In total approximately 7.1 (w/v)% rice flour and 0.86 (w/v)% sodium nitrate is recommended for phytase production by the selected strain. The maximal activity is achieved on the 6^{th} day of fermentation. Based on the model raising the concentration of rice flour with optimal $NaNO_3$ concentration – results in an increased enzyme activity, but the concentration of rice flour cannot be further increased in a submerged fermentation process because of the high viscosity of the medium.

The different initial pH had no significant effect on enzyme production therefore adjusting pH was not necessary. All fermentation media were prepared with distilled water.

During optimization of the inoculum size, the highest phytase activity was assayed when 150 ml of fermentation medium in 1000 ml flask was cultured with 5% by volume of

inoculum. Despite of that the surfactants at proper concentration are generally able to promote excretion of enzyme, in the case of *A. niger* strain F00735 this effect was not significant, and what is more an inhibitory effect was observed when concentration of these compounds were increased. Based on these results during the fermentation the supplementation of surfactant is not necessary.

The purification procedures were performed in combination of different chromatographic steps after fractioned precipitation by ammonium sulphate. Two different enzyme proteins were obtained namely phytase I and II. The yield of the phytase I and the phytase II was 10.5% and 4.8 %, and purification was fold 21 and 4.7, respectively.

The molecular weight of phytase I was approximately 117-120 kDa and that of phytase II was 65-67 kDa. The pH optimum of phytase I is pH=5.0 that is in agreement with that of *Aspergillus* species. In the case of phytase II strong acidic optimum pH (pH=2.5-3.5) was determined. Both phytases have maximal activity at 60°C. The half-life times of phytase I and II at 60°C were 1.5 hours and 54 hours, respectively. The activity of phytase II did not decrease below 50 % after 2 months at 50 °C.

The effect of metal ions on enzyme activity was investigated. The enzyme activity of phytase II was less modified by metal ions, than phytase I. In the case of phytase I, the Mg^{2+} and Mn^{2+} ions at concentration of 5 mM increased the enzyme activity in more than 2.5 times. 5 mM Cu^{2+} ions had inhibitory effect on enzyme activity of phytase II.

Summary

Based on the comparison of the properties of two enzymes from different origins (Table 1.) it can be established that the application of a *T. lanuginosus* IMI 096218 strain resulted shorter fermentation time (2-4 days) than the *A. niger* strain F00735 (6-7 days). The time required to propagate the inoculum culture in both cases were two days. By increasing the concentration of rice flour to 7.1% the enzyme activity of *A. niger* phytase was enhanced, while in case of *T. lanuginosus* 5% of rice flour was optimal. It is not necessary to adjust the initial pH or apply surfactant during fermentation by *A. niger.*

In both cases of *Aspergillus* and *Thermomyces,* homogenous enzyme proteins were obtained after 4 and 3 chromatographical steps following precipitation, respectively. The optimal temperature is higher (70 °C) in the case of thermophilic than of mesophilic (60 °C) fungus. At the optimum temperature, 60 °C, the half-life times of phytase from *T. lanuginosus* and the phytase I from *A. niger* were the same (1.5 hours). The half-life time of the phytase II

was 54 hour at 60°C, exhibiting high stability. The other advantage of this enzyme is the optimum pH that was in the strong acidic range of pH=2.5-3.5.

Table 1. Comparison of fermentation technologies, purification and characterization of phytase enzymes originating from *A. niger* and *T. lanuginosus*

FERMENTATION TECHNOLOGY			
	***Aspergillus niger* F00735**		***Thermomyces lanuginosus* IMI 096218**
Time and quantity of inoculum	2 days, 5 (w/v)%		2 days (40 hour), 5 (w/v) %
Time of fermentation	6-7 day		1-2 day
Fermentation conditions	28°C, 220 rpm		47°C, 220 rpm
Carbon source	7.1% rice flour		5% rice flour
Nitrogen source	0.86% $NaNO_3$		0.86% $NaNO_3$
Initial pH	no pH adjust needed (pH=5.3)		Tris-maleate/NaOH buffer pH=7.5
Surfactant	-		0.1% Tween 40
Maximal activity	2000 U/l		2000 U/l
PURIFICATION OF ENYZME			
Precipitation	ammonium sulphate fractionation		up to 80% saturation of ammonium sulphate
Chromatographic steps	ion-exchange chromatography		hydrophobic interaction chromatography
	gelfiltration		hydrophobic interaction chromatography
	chromatofocusing		ion-exchange chromatography
	hydrophobic interaction chromatography		
CHARACTERIZATION OF ENZYME			
	Phytase I	Phytase II	Phytase
Molecular weight	117-120 kDa	65-67 kDa	60 kDa
Optimal temperature	60°C		70°C
Optimal pH	5.0	2.5-3.5	5.5, 7.5
Half-life time $t_{1/2}$ (60 °C)	1.5 h	54 h	1.5 h
Half-life time $t_{1/2}$ (55 °C)	48 h	120 h	2.25 h
Activator (5 mM)	Mg^{2+}, Mn^{2+} Ca^{2+}, Co^{2+}		K^{+}, Ca^{2+}, Mg^{2+} Fe^{2+} (1 mM), Fe^{3+} (1 mM)
Inhibitor (5 mM)		Cu^{2+}	Cu^{2+}, Zn^{2+}, Ag^{+}, Co^{2+}

Also in acidic range (pH=5.0-5.5) is the pH optimum of most phytases and it is typical property of both fungi. The Ca^{2+} and Mg^{2+} ions in concentration of 5 mM activate both phytases from *T. lanuginosus* and phytase I from *A. niger.*

Altogether it can be concluded that both *A. niger* F00735 and *T. lanuginosus* IMI 096218 strains secreted appreciable level of extracellular phytase enzyme on rice flour substrate when submerged fermentation is performed. These enzymes exhibited different physico-chemical properties and kinetic parameters putting them to high potential to develop enzyme technology for industrial applications such as food and feed, as well as pharmaceutical industries.

New scientific achievements

1. Fourteen thermophilic *Thermomyces lanuginosus* filamentous fungi strains were screened for their ability to produce extracellular phytase. *T. lanuginosus* IMI 096218 proved to be the best one, and a fermentation technology was developed for phytase enzyme production on laboratory scale. Effects of different natural substrates containing phytate such as soy flour, wheat bran, corn flour, rice flour on phytase production were investigated and the highest extracellular activity was achieved on 5% rice flour after initiation with 5% of inoculum. It can be concluded that the enzyme activity increased when the medium was prepared with Tris-maleate/NaOH buffer (pH=7.5) and the enzyme yield maximised in the 2^{nd} day of the fermentation. Addition of 0.1% Tween 40 surfactant to the medium enhances the phytase secretion (BUJNA et al. 2011).

2. Eleven mesophilic *Aspergillus* strains were screened for their ability to produce extracellular phytase. The highest phytase activity was achieved by *Aspergillus niger* F00735 and a fermentation technology was developed for phytase enzyme production on laboratory scale. Different natural substrates – rice flour, pea flour, corn flour, corn starch, corn grit, soy flour, wheat flour and wheat grit – were examined in respect of phytase productivity. The rice flour proved to be the best substrate. The Central Composite Design was used to determine the optimal concentration of the main component of fermentation medium. Applying 0.86% sodium-nitrate and 7.1 % rice flour without adjusting the initial pH of fermentation medium resulted in threefold increase of enzyme activity (BUJNA et al. 2013).

3. Extracellular phytase from *Thermomyces lanuginosus* IMI 096218 strain was purified to homogeneity. Two protein bands with a molecular weight of approximately 60 kDa and 90

kDa were detected by SDS polyacrylamide gel electrophoresis. It can be assumed that the enzyme is multimer. The main properties of the purified enzyme: pH and temperature optima are pH=5.5 and pH=7.5, and 70 °C, respectively. The presence of 1 mM Fe^{2+}, Fe^{3+} and 5 mM K^{+}, Ca^{2+} and Mg^{2+} ions proved to be activator, whereas the presence of 5 mM Cu^{2+}, Zn^{2+}, Ag^{+} and Co^{2+} ions strongly inhibited the enzyme reaction. Kinetic parameters on sodium-phytate substrate were determined by linear Lineweaver-Burk plot: K_M=0.285 mM, v_{max}=0.126 mM/min, and Hanes-Woolf plot: K_M=0.312 mM, v_{max}=0.132 mM/min. The half-life time of enzyme at temperature 54-58 °C and pH range 5.0-7.5 is more than 100 min.

4. Extracellular phytase from *Aspergillus niger* F00735 strain was purified to homogeneity. Two different enzyme proteins were obtained (phytase I and II). The molecular weight of proteins was approximately 117-120 kDa (phytase I) and 65-67 kDa (phytase II). The pH optimum of phytase I and phytase II are pH=5.0 and pH=2.5-3.5, respectively. Both phytases have maximal activity at 60°C. The half-life times of phytase I and II at 60°C were 1.5 hours and 54 hours, respectively. The activity of phytase II did not decrease below 50 % after 2 months at 50 °C. The enzyme activity of phytase II was less modified by metal ions, than of phytase I. In the case of phytase I, the Mg^{2+} and Mn^{2+} ions at concentration of 5 mM increased the enzyme activity.

4 Recommendation for further research

Characteristics, catalytical and physico-chemical properties of different phytases are determined by the organism they originate from. Due to the diversity of microorganisms the microbial phytases have different properties, thus there are rich sources of enzymes with proper environmental parameters for industrial applicability. The phytase preparation, which improves the bioavailability of nutrient, is expected to exhibit resistance to the acidic pH of stomach and digestive enzymes, thus hydrolyse phytate-P in the digestive tract. Furthermore it should be stable enough to resist inactivation by heat during feed pelleting and maintain their activity.

Phytase II enzyme from *A. niger* F00735 strain is promising for feeding purposes because its pH optimum is in the range of 2.5-3.5, and its half-life time is 2 months at 50°C. Application of phytase in human medicine is a developing area. Phytases hydrolyze phytic acid through a series of myo-inositol phosphate intermediates, which has been shown to have beneficial physiological effect. Numerous studies in the medical literature have reported myo-inositol phosphate intermediates mainly myo-inositol-triphosphate as a broad-spectrum anti-

neoplasmic agent. Different types of phytases can be classified as 3-phytase, 4/6 phytase and 5-phytase according to their breakdown mechanism, which results myo-inositol-phosphate with various structure. Phytases originating from fungi are typically classified as 3-phytase but my achievements are not sufficient to classify the purified enzymes.

For further development design of bioconversion experiments can be recommended. Based on the analysis of the hydrolysis products, the mechanism of the given enzyme can be determined. After scale up each intermediates can be purified by preparative HPLC technology, and their structure by NMR techniques can be determined. Position and presence of phosphorous groups provides information about the first cleavage site and sequential hydrolysis of phosphate esters, on the basis which the enzyme are classified. This knowledge makes possible the medical testing of myo-inosytol phosphate bioconversion products .

Based on our results, significant differences were discovered among phytases originating from *A. niger* and *T. lanuginosus.* Considering that numerous references are available in the scientific literature about *A. niger* phytase, it would be important to perform such studies on phytases produced by *Thermomyces*.

5 Publications in the field of the dissertation

Articles in journals

Journals with impact factor, foreign language

Bujna, E., Kukovics, F., Nguyen, Q.D., Rezessy-Szabó, J.M. (2013). Rice flour as potential carbon source for production of phytase by *Aspergillus niger*. Acta Alimentaria. 42: 1-9

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Book chapter

Q. D. Nguyen, **E. Bujna**, Á. Hoschke, J. M. Rezessy-Szabó. 2011. Thermophilic Fungus *Thermomyces lanuginosus*: Current Review on Potential Source for Thermostable Enzymes. Chapter II. In: Biotechnology of Microbial Enzymes. Editors: Vijai Kumar Gupta and Manimaran Ayyachamy. Nova Science Publishers, Inc.ISBN: 978-1-62100-131-7, pp 21-56