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Formation of *myo*-Inositol Phosphates by *Aspergillus niger* 3-Phytase

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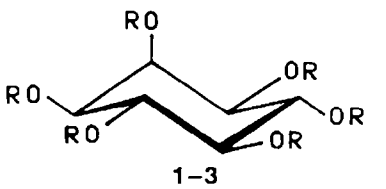
ABSTRACT. Kinetics of phytate hydrolysis by *Aspergillus niger* phytase and correlation between the amount of released phosphate and creation of lower *myo*-inositol phosphates were investigated. Phytase was able to hydrolyze *myo*-inositol hexakis-, pentakis-, tetrakis-, and trisphosphates. Finally, about 56 % of total phosphate were released and *myo*-inositol bisphosphate was detected as the end-product.

myo-Inositol (1) — the most common inositol isomer in nature — is present mostly in the form of inositol phospholipids as the component of cell membranes in animals and microorganisms. In plants it occurs mostly in the form of *myo*-inositol hexakisphosphate (phytic acid, IP_6; 2) and its Ca^{2+}, Na^+, and K^+ salts (phytates) where it serves, in the seeds, as the major phosphate store (Billington 1993). In addition, other physiological functions of phytic acid in plants were suggested, such as energy storage, ATP competition, complexation to multivalent cations, and regulation of the inorganic phosphate level (Billington 1993).

Besides IP_6, also the lower phosphoric esters of *myo*-inositol (mono-, bis-, tris- and tetrakisphosphates) occur frequently in nature. They play a crucial role in transmembrane signaling processes and in calcium mobilization from an intracellular store in animal as well as in the plant tissues (Michell 1975; Berridge and Irvine 1984; Samanta *et al.* 1993; Dasgupta *et al.* 1996; Kryštofová *et al.* 1998). Recently, it was suggested that a phosphoinositide-signalling system is also involved in some eukaryotic microorganisms (Van Haastert 1989; White 1991; Irvine 1992). The biochemistry of all *myo*-inositol phosphates and their position in cell regulation processes, as well as the role of the inorganic phosphate level and its regulation is not fully understood.



1–3

	R	
1	H	*myo*-inositol
2	$-PO_3H_2$	*myo*-inositol hexakisphoshate (phytic acid)
3	$-PO_3Na_2$	dodecasodium phytate

Not only intense metabolic investigations but also the possibility to use *myo*-inositol phosphate derivatives as enzyme stabilizers (Siren 1986) or enzyme inhibitors and hence potential drugs (Laumen and Ghisalba 1994) resulted in the need of various *myo*-inositol phosphates preparations.

6-Phytase (*myo*-inositol-hexakisphosphate 6-phosphohydrolase, EC 3.1.3.26) hydrolyzing phytic acid (2) and its salts to the lower phosphoric esters is of utmost importance for the *myo*-inositol phosphates turnover. This enzyme (*for review see* Dvořáková 1998) is mainly used in fodder industry for increasing the nutritive value of fodder by degradation of phytic acid which is considered to be an antinutritive part of the diet of monogastric animals (Newman 1991). Recently, it was suggested to employ phytase for preparation of some lower *myo*-inositol phosphates (Phillippy *et al.* 1987) as an attractive alternative to the chemical synthesis, demanding selectively protected inositol intermediates presenting very often fundamental problems.

Investigation of pathways of enzymic phytate hydrolysis (Lim and Tate 1973; Zyla 1994; Barnentos *et al.* 1994; Van Der Kaay and Van Haastert 1995; Tseng *et al.* 2000) indicate that both the intermediates and the end products depend on the type and source of phytase. Hence the hydrolysis of phytate by phytase is attractive for both basic and industrial research.

Extracellular 3-phytase (*myo*-inositol-hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8) produced by *Aspergillus niger* strain 92 at high levels (Volfová *et al.* 1994) was purified and characterized (Dvořáková *et al.* 1997), and its effect in fodder mixture on phosphorus and calcium utilization in chicken was investigated (Zobač *et al.* 1997). In this communication we report on the kinetics of phytate hydrolysis by *A. niger* 3-phytase, the fundamental parameter necessary for designing large-scale production of lower *myo*-inositol phosphates.

MATERIAL AND METHODS

Enzyme. 3-Phytase produced by *A. niger* strain 92 (Volfová *et al.* 1994) was prepared and purified according to Dvořáková *et al.* (1997).

Enzymic hydrolysis of dodecasodium phytate (**3**). The hydrolytic reaction was carried out at 40 °C in the mixture (100 mL) consisting of 50 mmol/L acetate buffer (pH 5), 1 mmol/L dodecasodium phytate and phytase (40 nkat/mL). The stability of both enzyme and dodecasodium phytate under the same reaction conditions were checked in tested control experiments with the enzyme or phytate alone. The samples (5 mL) were assayed for inorganic monophosphate and the spectrum of lower *myo*-inositol phosphates generated from IP_6.

Separation of myo-*inositol phosphates. myo*-Inositol phosphates were separated by HPLC using Mono Q HR 5/5 column (*Pharmacia Biotech AB*, Uppsala, Sweden) according to Freund *et al.* (1992) with few modifications. After the sample (2 mL) injection the column was washed with water (10 mL) to elute free *myo*-inositol out. Inorganic monophosphate and *myo*-inositol phosphates were consecutively eluted with ammonium formate by the following stepwise program (flow rate 1 mL/min): (*1*) linear gradient 0–195 mmol/L (2 mL); (*2*) isocratic elution with 195 mmol/L (3 mL); (*3*) linear gradient 195–950 mmol/L (44 mL).

The concentration of liberated inorganic monophosphate was determined using ammonium molybdate method (Watanabe and Olsen 1965).

Detection of myo-*inositol phosphates* (IP_n). The *myo*-inositol phosphates in the collected fractions were determined colorimetrically (Zyla 1991) using the Wade reagent (0.03 % $FeCl_3 \cdot 6H_2O$ and 0.3 % 5-sulfosalicylic acid). Phosphate esters of *myo*-inositol form complexes with ferric ion having similar Fe/P ratios at pH 4.0. The collected eluate (200 μL) and Wade reagent (200 μL) were added to the acetate buffer (600 μL, 125 mmol/L, pH 4.0). Absorbance (A_{500}) was corrected against the blank (containing water instead of eluate). Standard measurements done with dodecasodium *myo*-inositol hexakisphosphate (**3**) and hexasodium *myo*-inositol 1,4,5-trisphosphate (*Sigma*) were used for calibration.

Identification of myo-*inositol phosphates.* Fractions of the separated peaks were combined and *myo*-inositol phosphates were identified both from the retention time of HPLC and by MS. Negative-ion electrospray (ESI) mass spectra were recorded on the double-focusing instrument Finnigan MAT 95 (*Finnigan MAT*, Bremen, Germany) of BE geometry equipped with the Finnigan atmospheric pressure ionization (API) ion source. The standard (IP_6) dissolved in the mobile phase (MeOH–H_2O 1:1) was continuously infused through a stainless steel capillary held at 3 kV into the ion source *via* a linear syringe pump at a rate of 30 μL/min (Harvard Apparatus 22). The lyophilizates of the collected HPLC fractions were redissolved in MeCN–H_2O (3:2, *V/V*) and ionized by the same technique. A mixture of polypropylene glycols (average M = 425 g/mol, *Aldrich-Chemie*, Steinheim, Germany) was used to calibrate the m/z scale of the mass spectrometer.

RESULTS AND DISCUSSION

Separation and characterization of phytate hydrolytic products

Mono Q HR 5/5 column used for separation of *myo*-inositol phosphates was first washed with water to elute free *myo*-inositol (**1**). No hydrolytic products were detected in the eluate. Inorganic monophosphate was eluted with 195 mmol/L ammonium formate, and IP_2, IP_3, IP_4, IP_5 and IP_6 were subsequently eluted at 300–360, 460–500, 650–700, 700–790, and 790–890 mmol/L ammonium formate, respectively (no IP_1 was detected). The nature of phosphates was characterized by electrospray MS. The deprotonated molecules were the most prominent ion species when the samples had been sprayed with a MeCN–H_2O mixture (Table I). Salty samples will generate much more complex negative ion mass spectra, *e.g.*, with the IP_6 (M = 923.6 g/mol) standard the corresponding ESI mass spectrum contains two envelopes (differing in Na^+ composition) of triply and doubly charged molecules (Fig. 1). The charge state of m/z 226 (3^+) can be derived from the number of peaks observed at the given nominal mass (Fig. 1, *inset*). The fragment PO_3^- ion is observed at m/z 79.

Kinetics of phytate hydrolysis

In a typical enzymic reaction 1 mmol/L dodecasodium phytate (**3**) was used to avoid phytase inhibition by higher substrate concentration (Dvořáková *et al.* 1997) and at the same time relatively low

phytase activity (40 nkat/mL of the reaction mixture) was employed aiming to follow phytate dephosphorylation step by step. The time course of dodecasodium phytate hydrolysis yielding the lower *myo*-inositol phosphates is shown in the Fig. 2; the amount of inorganic monophosphate simultaneously being released from IP_6 is given in Fig. 3. At the beginning of the reaction — after 10, 20, and 30 min (when 4, 8, and 16 %, respectively, of the total phosphate were split – only *myo*-IP_5 and *myo*-IP_4 were identified as the hydrolytic products. Later, after 2–3 h when 37–43 % of the total phosphate were released, *myo*-IP_3 and *myo*-IP_2 prevailed in the hydrolytic products, and *myo*-IP_5 and *myo*-IP_4 were present as minor components, no *myo*-IP_6 being identified at this time. After 4 h, 47 % of total phosphate was released, and the reaction yielded *myo*-IP_2 and *myo*-IP_3. Hydrolysis rate obviously decreased with reaction time (Fig. 3) and after 1 d 56 % of total phosphate was released and hydrolysis was stopped (*data not shown*) although phytase remained active. The enzymic hydrolysis of phytate catalyzed by *A. niger* 3-phytase yielded *myo*-IP_2 as the end product, no *myo*-IP_1 and free *myo*-inositol (1) were detected in the reaction medium.

There are at least two major factors influencing the course and the end products of this reaction. First, the affinity of the phytase to the *myo*-inositol phosphates decreases with lowering the degree of phosphorylation of the *myo*-inositol molecule. Therefore, its ability to hydrolyze *myo*-P_2 is extremely low or none. Second, the enzyme is inhibited by inorganic phosphate released during the reaction. Although inorganic phosphate is only a weak noncompetitive inhibitor of phytase (K_i = 2.85 mmol/L; Dvořáková *et al.* 1997) and its concentration in the reaction mixture being 3.36 mmol/L after 1 d should decrease the rate of hydrolysis approximately only twice, it is necessary to note that K_i was estimated for the IP_6 hydrolysis.

Table I. *m/z* values of the $[M-H]^-$ ions of *myo*-inositol phosphates detected by negative-ion electrospray mass spectrometry

Compound	*m/z* $(M-H)^-$	
	calculated	measured
IP_2	339.0	339.0
IP_3	419.0	418.9
IP_4	498.9	498.8
IP_5	578.9	578.8
IP_6	658.9	658.7

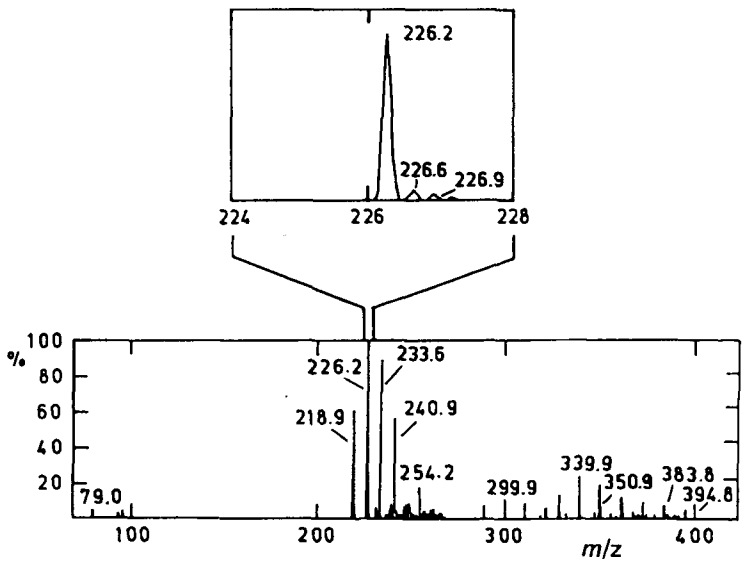


Fig. 1. Negative-ion electrospray mass spectrum of dodecasodium phytate (3; M = 923.6 g/mol); *inset*: detail of triply-charged molecular ion species.

The same P_i concentration can influence the kinetics of the hydrolysis of lower *myo*-inositol phosphates more significantly. Therefore, most probably a combination of both factors, *i.e.*, low affinity to lower *myo*-inositol phosphates and phytase inhibition with P_i, yielded a substantial drop of the reaction.

Such a course of IP_6 hydrolysis is similar to hydrolytic pathways of IP_6 catalyzed by phytase from *Aspergillus ficuum* (Ullah and Phillippy 1988) and that from *Escherichia coli* (Greiner and Konietzny 1996) that yielded also IP_2 as end products. On the other hand, *Bacillus subtilis* phytase dephosphorylated phytate down to IP_1 (Powar and Jagannathan 1982) and phytase produced by *Saccharomyces cerevisiae* yielded mainly IP_3 (Zyla 1994). This diversity can be caused by different kinetic

constants (K_m for IP_6-IP_1; inhibition by excess of IP_6; and inhibition by P_i) of phytases from different sources.

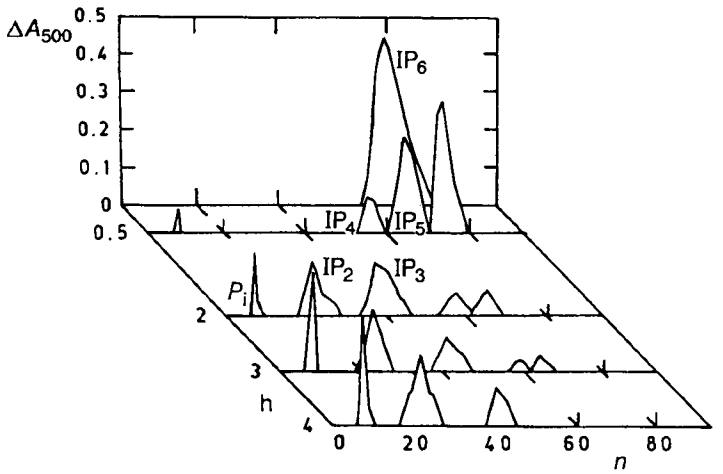


Fig. 2. Elution profiles of *myo*-inositol phosphates formed under dephosphorylation of dodecasodium phytate by *A. niger* phytase.

Extracellular *A. niger* 3-phytase consequently hydrolyzing phytate to IP_5-IP_2 can be used for preparation of lower *myo*-inositol phosphate derivatives. The rate of enzymic phytate hydrolysis can be controlled by the amount (activity) of phytase and/or by the reaction temperature due to the remarkable thermostability of this enzyme (Dvořáková *et al.* 1997). Very fast and easy estimation of P_i and correlation between the amount of released P_i and generation of lower *myo*-inositol phosphates can indicate products prevailing in the reaction mixture.

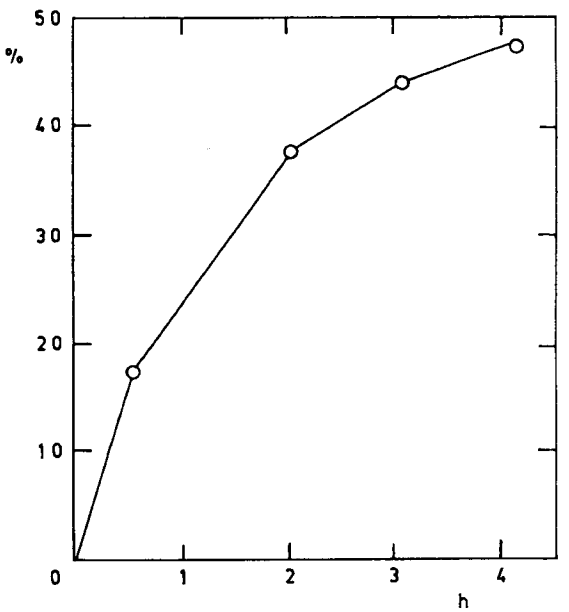


Fig. 3. Inorganic phosphate released from dodecasodium phytate (3) by *A. niger* phytase.

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