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PURIFICATION AND CHARACTERISATION OF AN EXTRACELLULAR PHYTASE FROM ASPERGILLUS TAMARI

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ABSTRACT

An extracellular phytase from *Aspergillus tamari* was purified about 51-fold to apparent homogeneity with a recovery of 20.3% referred to the phytase activity in the crude extract. Purification was achieved by ammonium sulphate precipitation, ion chromatography and gel filtration. The purified enzyme behaved as a monomeric protein with a molecular mass of about 85 kDa and exhibited maximal phytate-degrading activity at pH 8.5. Optimum temperature for the degradation of phytate was 28°C. The kinetic parameters for the hydrolysis of nitro phenyl phosphate disodium salt were determined to be Km = 54 $\mu mol^{-1}$and kcat = 190 sec^{-1} at pH 8.5 and 28°C. The purified enzyme was rather specific for phytate dephosphorylation. It was shown that the phytase preferably dephosphorylates *myo*-inositol hexakisphosphate in a stereospecific way by sequential removal of phosphate groups via D-Ins(1,2,4,5,6)P5, D-Ins(1,2,5,6)P4, D-Ins(1,2,6)P3, D-Ins(1,2)P2 to finally Ins(2)P

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KEY WORDS

Aspergillus tamari, phytate-degrading enzyme, phytate, phytase.

INTRODUCTION

During food processing and digestion, phytate can be partially dephosphorylated by phytate-degrading enzymes (phytases) to yield a large number of positional isomers of *myo-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphates*. The number and distribution of the phosphate residues on the *myo-inositol* ring determine the metabolic effects triggered by the individual *myo-inositol* phosphate isomer. Different phytases [*myoinositol(* 1,2,3,4,5,6) hexakisphosphate phosphohydrolases] may exhibit different phytate degradation pathways and therefore lead to the generation and accumulation of different *myo-inositol* phosphate intermediates. Phytate-degrading activity has been detected in plants, micro-organisms, and in some animal tissues (3, 6) and phytases from several plant and microbial species have been purified and characterized (5, 7). For the phytase of *Aspergillus tamari* a dual phytate dephosphorylation pathway resulting in the generation of two *myo-inositol trisphosphates* was reported (1,2,3).

Aims & Objectives

A phytase from *Aspergillus tamari* was isolated, purified, characterized and its enzymatic properties including the pathway of phytate dephosphorylation was determined.

METHODS

Sample Collection

Samples used for isolation of Phytase producing organisms were organic soil, fruits and vegetables. Among fruits, riped fruits of citrus like lemon and orange have been used for isolation purpose which give more diversifying fungal growth. Different vegetables like tomato and potato have been used but tomatoes give more diversifying Phytase producing organisms. Riped strawberries also have been used which show more types of fungal indices.

Screening the Organism

The samples were plated on the common media and the fungi were screened from all different types of fungi. It was further isolated on the Czapex dox agar media. Plates were subjected to incubation and different species were isolated. *Aspergillus spp.* was selected. Biomass was collected from the plates and was inoculated in the Phytase producing medium with substrate 10 mM 4-nitro phenyl phosphate disodium salt as substrate. Biomass collected on 1500 rpm after sufficient incubation, filtration and centrifugation, crude enzyme is used for further studies.

Enzyme assay

Phytase activity was calculated by measuring the rate of increase of inorganic orthophosphate according to the method of Fiske and Subbarow (1925).
The protein estimation was carried out by Folin-Lowry's method.

Enzyme purification and Extraction

The crude enzyme obtained after the production was then purified by DEAE-cellulose column chromatography. Before going for column chromatography enzyme purification was done by using ethanol. Enzyme production was carried out on a rotary shaker at 180 rpm and 30°C for 1 h using 150 ml of 0.1 M acetate buffer, pH 5.0 per 40 g of fermentation medium. As this enzyme happened to be extracellular enzyme, particulate material was removed by filtration and the clear extract was submitted to

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precipitation with 0-90% ammonium sulfate and centrifuged at 5000 rpm for 30 min at 4°C.

Standard phytase assay

Phytase activity was determined at 37°C in 350 µl of 100 mM sodium acetate buffer (pH 5.0) containing 875 nmol sodium phytate. The enzymatic reaction was started by adding 10 µl of enzyme solution to the assay mixture. After incubating for 30 min at 37°C, the liberated phosphate was measured according to the ammonium molybdate method with some modifications. Blanks were run by addition of the ammonium molybdate solution prior to addition of the enzyme solution to the assay mixture. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of inorganic phosphate per minute under the assay conditions. To determine the substrate selectivity of the purified phytase, several phosphorylated compounds in addition to phytate were used for Km and Vmax estimation. The enzyme system consisted of 350 µL 0.1 M sodium acetate buffer, pH 5.0, containing the phosphorylated compound in a serial dilution of a concentrated stock solution (10 mM). The enzymatic reactions were started by adding 10 µl of enzyme to the assay mixtures. The rate of reaction was linear for the 30 min incubation time. The kinetic constants (Km, Vmax) were calculated from Lineweaver-Burk plots of the data. To study the pH-optimum and the pH-stability of the purified phytase, the following buffers were used in the above described standard assay: pH 1.0-3.5, 0.1 M glycine-HCl; pH 3.5-6.0, 0.1 M sodium acetate-HCl; pH 6.0-7.0, 0.1 M Trisacetic acid; pH 7.0-9.0, 0.1 M Tris-HCl; pH 9.0-10.0, 0.1 M glycine-NaOH.

Preparation of myo-inositol pentakis- and trisphosphate isomers

50 µmol *myo*-inositol hexakisphosphate in the corresponding incubation buffer were incubated at 37°C with 0.4 U of the phytases in a final volume of 20 ml. After an incubation period of 60 minutes (*myo*-inositol pentakisphosphate preparation) or 8 hours (*myo*-inositol trisphosphate preparation), respectively, the reactions were stopped by heat treatment (95°C, 10 min). The incubation mixtures were lyophilised and the dry residues were dissolved in 10 ml 0.2 M ammonium formate, pH 2.5. The solutions were loaded onto a Q-Sepharose column (2.6 x 90 cm) equilibrated with 0.2 M ammonium formate, pH 2.5 at a flow rate of 2.5 ml min^{-1}. The column was washed with 500 ml of 0.2 M ammonium formate, pH 2.5; the bound *myo*-inositol trisphosphates were eluted with a linear gradient from 0.2 to 0.6 M ammonium formate, pH 2.5 (1000 ml) and the bound *myo*inositol pentakisphosphates with a linear gradient from 1.0 to 1.4 M ammonium formate, pH 2.5 (1000 ml) at 2.5 ml min-1. 10 ml fractions were collected. From even-numbered tubes, 100 µl aliquots were lyophilised. The liberated phosphate was quantified by a modification of the ammonium molybdate method (Heinonen, J.K.; Lahti, R.J. (1981)). The content of the fraction tubes corresponding to the *myo*-inositol tris- and pentakisphosphates, respectively, were pooled and lyophilised until only a dry residue remained. Ten milliliters of water were used to redissolve the residues. Lyophilisation and redissolving were carried out repeatedly twice for complete removal of ammonium formate. *Myo*-inositol phosphate concentrations were determined by HPLC ion-pair chromatography on Ultrasep ES 100 RP18 (2 x 250 mm). The column was run at 45°C and 0.2 ml min^{-1} of an eluant consisting of formic acid:methanol:water:TBAH (tetrabutylammonium hydroxide) (44:56:5:1.5 v/v), pH 4.25, as described by Sandberg and Ahderinne (Sandberg, A.-S.; Ahderinne, R. (1986)). A mixture of the individual *myo*-inositol phosphate esters (InsP3 - InsP6) was used as a standard. The purity of the *myo*-inositol phosphate preparations was determined on a High-Performance Ion Chromatography system (Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. (1998)).

Production of enzymatically formed hydrolysis products

The enzymatic reaction was started at 37°C by addition of 50 µl of a suitable diluted solution of the phytase from *Aspergillus niger* 11T53A9 to the incubation mixtures (1 U ml^{-1}). The incubation mixture consisted of 1250 µl 0.1 M sodium acetate buffer, pH 5.0 containing 3.125 µmol sodium phytate. From the incubation mixture, 150 µl samples were removed periodically and the reaction was stopped by heat treatment (95°C, 10 min). For the identification of phytate degradation products, 30 of the heat-treated samples were chromatographed on a High-Performance Ion Chromatography system (Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. (1998)).

Identification of enzymatically formed hydrolysis products

Myo-Inositol phosphate isomers were determined and separated on a HPIC system using a Carbo Pac PA-100 (4 x 250 mm) analytical column and a gradient of 5–98% HCl (0.5 M, 0.8 ml min^{-1}) (Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. (1998)). The eluants were mixed in a post-column reactor with 0.1% Fe(NO3)3 x 9 H2O in a 2% (v/v) HClO4 solution (0.4 ml min^{-1}) (Phillippy, B.Q.; Bland, J.M. (1988)). The combined flow rate was 1.2 ml min^{-1}.

Quantification of the liberated phosphate

The liberated phosphate was quantified by a modification of the ammonium molybdate method (Heinonen, J.K.; Lahti, R.J. (1981)). 1.5 ml of a freshly prepared solution of acetone:2.5 M sulfuric acid:10 mM ammonium molybdate (2:1:1 v/v) and thereafter 100 µl 1.0 M citric acid were added to 400 µl of the suitably diluted hydrolysis mixtures or to the mixtures of the phytase assay. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. In order to quantify the released phosphate a calibration curve was produced over the range of 5 to 600 µmol phosphate.

Identification of the myo-inositol monophosphate isomer

Myo-inositol monophosphates were produced by incubation of 1.0 U of the phytase from the purified phytase with a limiting amount (0.1 µmol) of phytate in a final volume of 500 µl of 50 mM ammonium formate. After lyophilisation, the residues were dissolved in 500 µl of a solution of pyridine:bis(trimethylsilyl)trifluoroacetamide (1:1 v/v) and incubated at room temperature for 24 h. The silylated products were injected at 270°C into a gas chromatograph coupled with a mass spectrometer. The stationary phase was methyl silicon in a fused silica column (0.25 mm x 15 m). Helium was used as the carrier gas at a flow rate of 0.5 m s^{-1}. The following heating program was used for the column: 100°C to 340°C, rate increase: 4°C min^{-1}. Ionisation was performed by electron impact at 70 eV and 250°C (Phillippy, B.Q.; Bland, J.M. (1988), Sandberg, A.-S.; Ahderinne, R. (1986)).

Purification of the phytate-degrading enzyme

The FPLC was run at 25°C and a flow rate of 1 ml min-1. Normal pressure chromatography was performed at 4°C (Phillippy, B.Q.; Bland, J.M. (1988), Sandberg, A.-S.; Ahderinne, R. (1986)).

Ammonium sulfate precipitation

The clarified enzyme extract was used for an ammonium sulfate precipitation at 0-90% saturation. The precipitate was suspended in 20 mM Tris-HCl buffer, pH 7.0 and dialyzed against the same buffer. Any insoluble material was removed by centrifugation.

DEAE-Sepharose CL 6B chromatography

The dialysed 90% ammonium sulfate precipitate was loaded onto DEAE-Sepharose CL 6B column (3 x 15 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.0. After eluting the unbound inactive protein from the column with equilibration buffer, a linear gradient of 0 to 0.5

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M NaCl (1000 ml) in 20 mM Tris-HCl buffer, pH 7.0 was applied. The fractions containing phytate-degrading activity were pooled and dialysed against 20 mM glycine-HCl buffer, pH 2.85 (Phillippy, B.Q.; Bland, J.M. (1988), Sandberg, A.-S.; Ahderinne, R. (1986)).

CM-Sepharose CL 6B chromatography

The dialysed pool from the previous step was loaded onto a CM-Sepharose CL 6B column (3.5 x 20 cm) equilibrated with20 mM glycine-HCl buffer, pH 2.85. The column was washed with 300 ml of the same buffer and then the proteins bound were eluted with a linear gradient from 0 to 1.0 M NaCl (1000 ml) in 20 mM glycine-HCl buffer, pH 4.5. The fractions containing phytate-degrading activity were pooled (Phillippy, B.Q.; Bland, J.M. (1988), Sandberg, A.-S.; Ahderinne, R. (1986)).

16/60 Sephacryl S-200 HR chromatography

The phytate-degrading enzyme-containing fractions from the previous step were loaded onto a 16/60 Sephacryl S-200 HR column equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl. The maximum loading volume per run was 2 ml. 2 ml fractions were collected. The fractions containing phytate-degrading activity were pooled and dialysed against 20 mM sodium acetate buffer, pH 5.0 (Phillippy, B.Q.; Bland, J.M. (1988), Sandberg, A.-S.; Ahderinne, R. (1986)).

Mono S HR 5/5 chromatography

The dialyzed fraction from the previous step was applied onto a Mono S HR 5/5 column equilibrated with 20 mM sodium acetate buffer, pH 5.0. The column was washed with equilibration buffer for 30 min and then with a linear gradient of 0 to 0.5 M NaCl in 20 mM sodium acetate buffer, pH 5.0, for 60 min. 2.5 ml fractions were collected. The fractions containing phytate-degrading activity were pooled (Phillippy, B.Q.; Bland, J.M. (1988), Sandberg, A.-S.; Ahderinne, R. (1986)).

Gel electrophoresis

The SDS-electrophoresis using 10% gels was performed according to Laemmli technique (Laemmli, U.K. (1970)). Gels were stained by Coomassie brilliant blue R-250.

Gel-filtration

To assess the molecular mass of the native phytase, the purified protein was gel-filtered on 16/60 Sephacryl S-200 HR equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl. The column was calibrated with glucose-6-phosphate dehydrogenase (Mr = 120,000), creatine kinase (Mr = 81,000), bovine serum albumin (Mr = 68,000), ßlactoglobulin (Mr = 40,000), and myoglobin (Mr = 17,000) (Laemmli, U.K. (1970)).

RESULT & DISCUSSION

Purification of the phytase

A summary of the purification scheme is given in Table 1. The phytase activity was eluted as a single sharp activity peak from each ion-exchange column after application of the gradient. A 51-fold purification of the enzyme was achieved with a recovery of 20.3%. The enzyme exhibited an activity of about 133Umg^{-1}.

Table 1

Purification scheme for the phytate-degrading enzyme from A. tamari

Purification step	Total protein(ug)	Total phytate degradation activity(U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	54,000	138	2.6	-	-
0-90% (NH4)2SO4 precipitation	26,539	117	4.4	1.7	84.8
DEAE Sepharose CL6B	5693	110	19.3	7.4	79.7
CM Sepharose CL6B	1156	62	53.6	20.6	44.9
Sephacryl S-200HR	287	36	125.4	48.2	26.1
MonoS HR5/5	211	28	132.7	51	20.3

Gel filtration of the native enzyme on a calibrated Sephacryl S-200 column gave a molecular mass of the phytase of 85,000 ± 2,500 Da with elution position being measured by determination of enzyme activity (figure 1).

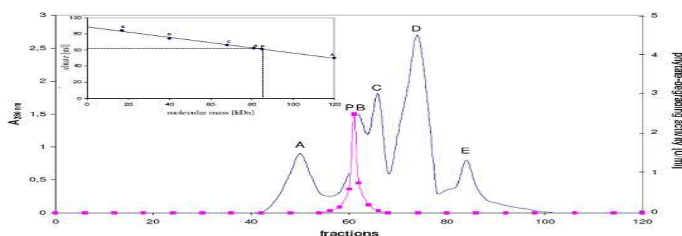

Fig.1 Estimation of the Molecular mass of the phytate degrading enzyme from A.niger by gel filtration
A; Glucose-6-phosphate (Mr=120,000) B: creatine kinase (Mr=81,000)
C: bovine serum albumin (Mr = 68,000), D: ß-lactoglobulin (Mr = 40,000),
and E: myoglobin (Mr = 17,000) P: phytate-degrading enzyme from *A.niger (Mr = approximately 85,000)*

Figure-1

This result indicates that the phytase could be regarded as homogeneous. According to the estimated molecular masses after SDS-PAGE, the protein band corresponds to a molecular mass of 85,000 Da. Consequently, this enzyme is a monomeric protein.

Identification of the myo-inositol monophosphates

Identification of the myo-inositol monophosphates formed was identified by gas chromatography. Fig. 2

mass spectrometry as Ins(2)P (data not shown).

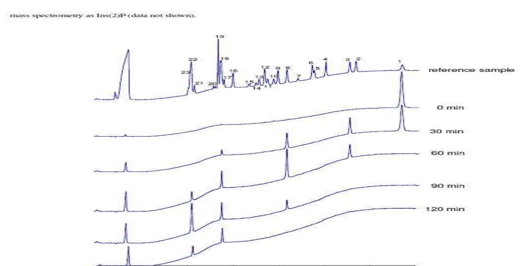

Figure 2. High performance ion chromatography analysis of hydrolysis products of phytate by an apparently pure phytate-degrading enzyme from *Aspergillus niger*

Reference sample: The source of the reference *myo*-inositol phosphates is as indicated in Skoglund *et al.* (23); Peaks: (1) Ins(1,2,3,4,5,6)P_6; (2) Ins(1,3,4,5,6)P_5; (3) D/L-Ins(1,2,4,5,6)P_5; (4) D/L-Ins(1,2,3,4,5)P_5; (5) Ins(1,2,3,4,6)P_5; (6) D/L-Ins(1,4,5,6)P_4; (7) Ins(2,4,5,6)P_4; (8) D/L-Ins(1,2,5,6)P_4; (9) D/L-Ins(1,3,4,5)P_4; (10) D/L-Ins(1,2,4,5)P_4; (11) Ins(1,3,4,6)P_4; (12) D/L-Ins(1,2,3,4)P_4; (13) D/L-Ins(1,2,4,6)P_4; (14) Ins(1,2,3,5)P_4; (15) Ins(4,5,6)P_3; (16) D/L-Ins(1,5,6)P_3; (17) D/L-Ins(1,4,5)P_3; (18) D/L-Ins(1,2,6)P_3, Ins(1,2,3)P_3; (19) D/L-Ins(1,3,4)P_3; (20) D/L-Ins(1,2,4)P_3, (21) D/L-Ins(2,4)P_2; (22) D/L-Ins(1,2)P_2, Ins(2,5)P_2, D/L-Ins(4,5)P_2; (23) D/L-Ins(1,4)P_2, D/L-Ins(1,6)P_2.

Figure-2

In order to determine the substrate selectivity of the purified phytase, several phosphorylated compounds (pNPP, phenylphosphate, glucose-1-phosphate, 1- naphthyl phosphate, 2-naphthyl phosphate, 2-glycero phosphate), in addition to phytate, were used for Km and Vmax estimation by detecting the release of the phosphate ion during hydrolysis using formation of a soluble phospho-molybdate complex in an acidic water-acetone mixture (table 2).

The kinetic parameters for the hydrolysis of phytate were determined to be Km = 54 μmol l-1 and kcat = 190 sec-1 at pH 5.0 and 37°C. Like other fungal phytases, the purified enzyme showed a substrate inhibition (3). The activity of the purified enzyme was inhibited at substrate concentrations > 2.5 mM at pH 5.0. The maximum amount of phosphate released from phytate by the purified enzyme suggests *myo-inositol* monophosphate as the final product of enzymatic phytate degradation.

Table-2 Kinetic constants for the hydrolysis of phosphorylated compounds by the phytate-degrading enzyme from *A.niger*

Substrate	K_M (μ M)	K_{cat} (sec^{-1})	K_{cat}/K_M ($s^{-1}M^{-1}$)
Phytate	54±1.4	190±4.3	3518519
p-nitrophyl phosphate	124±3.9	35±0.9	282258
1-naphthyl phosphate	247±6.7	23±1.5	93117
2-naphthyl phosphate	276±7.1	22±1.3	79710
2-glycero phosphate	521±9.1	9±0.3	17274
Glucose-1-phosphate	741±6.4	6±0.4	8097
Phenyl phosphate	177±1.3	36±1.7	203390

DISCUSSION

The enzymatic properties of the purified phytase suggest that this enzyme is very similar to the *Aspergillus tamari* phytases reported in the literature (8, 9, 10, 11, 12, 13, 14, 15, 16). The subunit molecular mass of the purified phytase was estimated to be 85 kDa by SDS PAGE. This molecular mass is in close agreement with that of the purified phytase (phy A) from *A. tamari kita.* Determination of the molecular mass of the biologically active enzyme was also carried out by gel filtration. A native molecular mass of 85 kDa was reported (figure 1), indicating that the catalytically active form of the enzyme is that
of a monomer. This is in accordance with all *A. tamari* phytases reported so far.
Substrate specificity studies (table 2) showed that the purified enzyme accepts p-nitro phenyl and phytate as a good substrate. All other compounds tested were only marginally hydrolysed by the purified enzyme. A detailed characterisation of the hydrolysis pathway of *myo*-inositol hexakisphosphate by the phytase from *Aspergillus tamari* purified to apparent homogeneity revealed that this enzyme dephosphorylates *myo*-inositol hexakisphosphate via D-Ins(1,2,4,5,6)P5, D-Ins(1,2,5,6)P4, D-Ins(1,2,6)P3, DIns(1,2)P2 to finally Ins(2)P. Therefore, this phytase has to be considered a 3-phytase (EC. 3.1.3.26). This is in agreement with the data reported by Ullah and Phillippy (28). In addition, Chen and Li (4) identified D-Ins(1,2,4,5,6)P5, D-Ins(1,2,5,6)P4, D-Ins(1,2,6)P3, D-Ins(1,2)P2 as phytate dephosphorylation products generated by a commercially available phytase derived from *A. niger*. In addition they found a further degradation

pathway proceeding from D-Ins(1,2,5,6)P4 via D-Ins(1,5,6)P3 to D-Ins(5,6)P2.

CONCLUSION

A detailed characterisation of the hydrolysis pathway of *myo-inositol hexakisphosphate* by the phytase from *Aspergillus tamari* purified to apparent homogeneity revealed that*,* this enzyme dephosphorylates *myo-inositol hexakisphosphate* via D-Ins(1,2,4,5,6)P5, D-Ins(1,2,5,6)P4, D-Ins(1,2,6)P3, DIns(1,2)P2 to .

finally Ins(2)P. Therefore, this phytase has to be considered as 3-phytase.

The formation of D-Ins(1,5,6)P3 upon action on phytate would require hydrolysis of the phosphate residue at position C-2 of the *myo-inositol* ring*.* This phosphate residue was shown to be resistant to dephosphorylation by phytases.

The only sequence of dephosphorylation which would be in agreement with the results obtained by high performance ion chromatography is C-3, C-4, C-5 and C- 6

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