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Purification and properties of a phytate-degrading enzyme produced by *Enterobacter sakazakii* ASUIA279

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ABSTRACT

An extracellular phytate-degrading enzyme produced by Enterobacter sakazakii ASUIA279 was purified to homogeneity using FPLC anion exchange chromatography and gel filtration. The enzyme was purified about 66-fold with a recovery of 27%. Its molecular mass was estimated to be 43 kDa by SDS-PAGE. The Michaelis constant (K_M) and turnover number (k_{cat}) for sodium phytate at pH 5.0 and 50°C were calculated from the Lineweaver-Burk plot to be 760 µM and 4.14s^{-1}, respectively. The enzyme showed narrow substrate specificity and not phytate, but GTP was dephosphorylated with the highest relative rate of hydrolysis. However, according to the k_{cat}/K_M values, phytate was concluded to be the in vivo substrate of the enzyme. Optimal activity was determined at pH 4.5 and 45-55°C. The enzyme was strongly inhibited by Fe^{3+}, Cu^{2+}, Zn^{2+}, molybdate, vanadate, fluoride and phosphate (1 mM).

Key-words: *Enterobacter sakazakii*; phytate-degrading enzyme; phytate, purification

INTRODUCTION

The interest in phytate-degrading enzymes and their application in the areas of nutrition, environmental protection, and biotechnology have advanced significantly over the past few years (Greiner, 2004). Phytases were originally proposed as animal feed additives to enhance the nutritional quality of plant material in feed for simple-stomached animals by liberating phosphate (Maga, 1982). Two-thirds of the phosphorus of feedstuffs of plant origin is present in the form of phytate (Nelson, 1967). Under dietary conditions, phytate phosphate could not be utilised by simple-stomached animals and its will dephosphorylated in the large intestine. The phosphate is not absorbed there and excreted in the faeces. This will contribute to the environment pollution problem in the form of phosphorus pollution to eutrophication of surface waters (Daly, 1991). The addition of phytase has seen as a way to reduce the level of phosphate pollution areas of intensive animal production, since the faecal phosphate excretion of these animals may be reduced by up to 50% (Walz and Pallauf, 2002) (see comment in the last sentence). Furthermore, the application of phytase in food area such as in food processing to produce functional food was binding to proteins and chelating minerals (Cheryan, 1980; Reddy et al., 1989). With the addition of phytase, the nutritional value of plant-based foods can be improved by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in the stomach or during food processing (Reddy et al., 1989; Sandberg and Andlid, 2002). Besides, certain myo-inositol phosphate derived from phytate by dephosphorylation has been proposed to have novel metabolic effects (Ohkawa et al., 1984; Potter, 1995; Vucenik and Shamsuddin, 2003).

Phytase activity has been detected in a variety of plant (Gibson and Ullah, 1988, Hübel and Beck, 1996), bacteria (Shimizu, 1992; Greiner et al., 1993; Yoon et al., 1996; Lan et al., 2002; Kim, 2003; Sajidan et al., 2004), fungi (Howson and Davis 1983; Ullah, 1988; Seguiella et al., 1993), yeast (Sano, 1999; Nakamura et al., 2000), protozoa (Van der Kaay and Van Haastert, 1995) and in some animal tissues (Maga, 1982). Phytases research and commercial production currently focuses on the soil fungus *Aspergillus* (Ullah,

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1988), *E.coli* (Greiner, 1993) and yeast. (not anymore also *E. coli* and yeast phytase are commercialised in the meantime). However, due to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases are a real alternative to the fungal enzymes. It is important to realise that any single phytase may never be able to meet the diverse needs for all commercial and environmental applications. Ongoing interest in screening micro-organisms and characterize the enzymes for novel and efficient phytases are needed. In this paper we report the purification and characterisation of an extra-cellular phytate-degrading enzyme produced by *Enterobacter sakazakii* ASUIA279 isolated recently from Malaysian maize plantation (Anis Shobirin et al., 2007) whose biochemical properties may render it of commercial interest.

MATERIALS AND METHODS

Culture and chemicals

Enterobacter sakazakii ASUIA279 was isolated from the endophyte zone of Malaysian maize (*Zea mays*) and identified by phenotypic (Anis Shobirin et al., 2007) and genotypic method (Anis Shobirin et al., 2009). The enzyme substrates were purchased from Merck (Darmstadt, Germany). Phytic acid, as a dodecasodium salt, was obtained from Sigma Chemical Co. (St. Louis, Mo.). Fast Flow CM Sepharose and Sephacryl S-200 were obtained from GE Healthcare (Piscataway, USA). All reagents were analytical grade.

Phytase production

Bacteria were cultured in LBRB medium containing per liter 5 g NaCl, 5 g yeast extract, 10 g peptone and 100g rice bran. The pH was adjusted to 7.0. The cells were grown aerobically at 37°C and agitated at 300 rpm.

Enzyme extraction

After 120 h of incubation, the enzymes were harvested by centrifugation at 10,000 rpm and 4°C for 15 min. The clear supernatant was adjusted to 80% ammonium sulfate to precipitate proteins. The precipitated protein material was collected by centrifugation and solubilized in 100 mM sodium acetate buffer pH 5 and extensively dialyzed against 50 mM sodium acetate pH 5. Any cloudiness was removed by centrifugation at 10,000 rpm and 4°C for 30 min.

Purification of phytate-degrading enzyme

FPLC was run at 25°C and a flow rate of 2 ml/min.

CM-Fast Flow sepharose chromatography

The dialyzed ammonium sulfate fraction was loaded onto a 10/10 CM-Fast Flow Sepharose column equilibrated with sodium acetate, pH 5. The column was washed with the same buffer and then the bound proteins were eluted with linear gradient from 0 to 1.0 M NaCl in 50 mM sodium acetate pH 5. The fractions containing phytase activity were pooled and dialyzed against 50 mM sodium acetate, pH 5.

Sephacryl S-200 HR chromatography

The dialyzed pool of the CM Fast Flow Sepharose was loaded onto a 16/100 Sephacryl S-200 HR column. The column was equilibrated and run with 50 mM sodium acetate, pH 5 containing 0.5 M NaCl. The fraction containing phytase activities were pooled.

Standard phytase assay

Phytate-degrading activity was determined at 50°C in 390 µl 100 mM sodium acetate buffer, pH 5.0 containing 1.03 mM sodium phytate. The enzymatic reaction was started by adding 10 µl of enzyme solution to the assay mixture. After incubating for 30 min at 50°C, the liberated phosphate was measured according to ammonium molybdate method (Heinonen and Lahti, 1981) with some modifications. Added to the assay mixture was 1.5 ml of a freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v) and 100 µl citric acid. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5-600 mmol phosphate ($\varepsilon = 8.7$ cm^2/nmol). Activity (units) was expressed as 1 µmol phosphate liberated per minute. Blanks were run by addition the ammonium molybdate solution prior to adding the enzyme to the assay mixture.

Protein determination

Total protein concentration was determined by the Coomassie blue G-250 dye-binding assay using bovine serum albumin as a standard (Bradford, 1976).

Molecular weight determination

The molecular weight of the purified enzyme was determined using SDS-PAGE (10% polyacrylamide gel). The purified protein was run along with low molecular weight markers (Pharmacia Biotech).

Phytate-degrading enzyme properties study

Substrate selectivity

To determine the substrate selectivity of the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279, several phosphorylated compounds in addition to phytate were used for K_M and V_{max} estimation. The incubation mixture consisted of 390 µl 0.1 M sodium acetate buffer, pH 5, containing the phosphorylated compound in a serial dilution of a concentrated stock solution (10mM). The enzymatic reactions were started by adding 10 µl of the enzyme to the assay mixtures. The incubation temperature is 50°C.

Effect of pH on enzyme activity

To study the pH-optimum and the pH-stability of the phytate-degrading enzyme, 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, 0.1 M sodium acetate-HCl; pH 6.0-7.0, 0.1 M Tris-acetate; pH 7.0-9.0, 0.1 M Tris- HCl; pH 9.0-10.0, 0.1 M glicine-NaOH. The pH stability of phytase was determined by subjecting it to pH 4.0 and 8.0 using 0.1 M sodium acetate and Tris maleate buffer, respectively for different time periods and then assay at 50°C using the standard assay.

Effect of temperature on enzyme activity

The temperature profile of the purified phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 was determined in the temperature range from 10 to 80°C using standard phytase assay. To check thermal stability, the purified enzymes was incubated at different temperatures, cooled to 4°C, and assayed using the standard phytase assay.

Effect of cations and potential inhibitors on enzyme activity

The effect of cations and potential inhibitors on enzyme activity was investigated by pre-incubating the compound with phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 for 15 min at 37°C before standard phytase assay was performed. The following cations and potential inhibitors were used in concentrations 0.1, 0.2, 0.5, 0.8 and 1.0 mM: Mg^{2+}, Ca^{2+}, Mn^{2+}, , Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Ag^{+}, Al^{3+}, 1,10-phenanthroline, EDTA, citrate, tartrate, molybdate, and vanadate. Fluoride and phosphate were used in the range 0.01-1.0 mM.

RESULTS AND DISCUSSION

Purification of the phytase

Enterobacter sakazakii ASUIA279 was isolated from the endophyte zone of Malaysian maize (*Zea mays*) as described previously (Anis Shobirin et al., 2007) and the bacterium was shown to synthesize an extra-cellular phytate-degrading enzyme. Generally, phytases produced by fungi are extra-cellular, whereas the enzymes from bacteria are mostly cell associated (Koneitzny and Greiner, 2004). The only bacteria showing extra-cellular phytase activity are those of the genera *Bacillus* (Choi et al., 2001; Kerovuo et al., 1998; Kim et al., 2003; Powar et al., 1982; Shimizu et al., 1992), *Lactobacillus amylovorus* (Sreeramulu, et al., 1996) and *Enterobacter* sp. 4 (Yoon et al., 1996). *Enterobacter sakazakii* ASUIA279 was fully identified using genotypic technique (Anis Shobirin et al., 2009).

The fermentation broth after 5 days of fermentation was used as the source of the enzyme. To purify the phytase, the crude proteins were concentrated by 80% ammonium sulfate precipitation. The phytate-degrading enzyme was purified using ion-exchange chromatography and gel filtration. A summary of the purification scheme is given in Table 1. The phytate-degrading enzyme was purified about 66-fold with a recovery of 27%. The enzyme exhibits a specific activity of about 2.03 U/mg.

Table 1. Purification scheme for the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude	336.0	10.4	0.031	1	100
0-80% $(NH_4)_2SO_4$	96.3	8.4	0.087	3	81
CM Sepharose FF	4.3	5.9	1.36	44	57
Sephacryl S-200 HR	1.4	2.8	2.03	66	27

Molecular properties

The molecular mass of the purified enzyme was estimated by SDS-PAGE. The phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 gave a single protein band upon electrophoresis after Coomassie staining of the gels (Figure. 1). The molecular mass of the denatured phytase was estimated to 42,800 ± 1100 Da. Phytate-degrading enzymes are high-molecular-weight proteins ranging from 40 to 500 kDa. The molecular mass of *Enterobacter sakazakii* ASUIA279 is similar to those from phytate-degrading enzymes purified from other bacterial sources, i.e. 40 kDa for *Klebsiella terrigena* (Greiner et al., 1997), 38-42 kDa for *Bacillus* sp. (Choi et al., 2001; Kerovuo et al., 1998; Kim et al., 1998; Shimizu, 1992), 42 kDa for *Escherichia coli* (Golovan et al., 2000; Greiner et al., 1993) and *Klebsiella pneumoniae* (Sajidan et al., 2004) and 47kDa for *Citrobacter braakii* (Kim et al., 2003). Since gel filtration was used to purify the enzyme also data about the molecular mass of the native enzyme should be available. Please present them to identify the phytase as a monomeric enzyme – furthermore, the SDS gels shows clearly that the enzyme is only partly purified. There are additional bands in the preparation which might interfere with characterization).

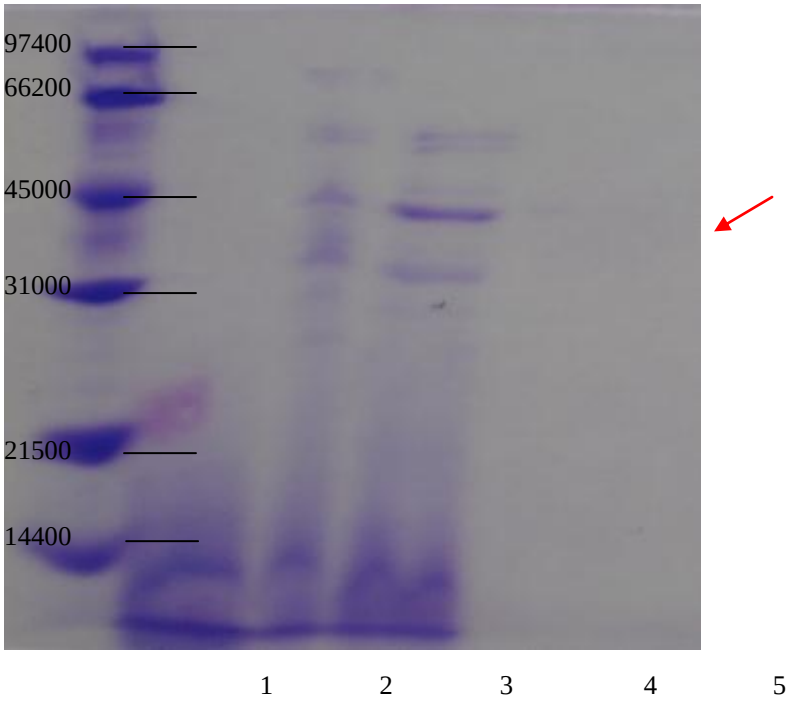

Figure 1- 10% SDS-PAGE of a preparation of the phytate-degrading enzyme from Enterobacter sakazakii ASFA279 stained with Coomassie Blue. Lane 1: Low Range SDS-PAGE Molecular Weight Standards, Lane 2: Crude Enzyme, Lane 3: Enzymes after Ammonium sulfate precipitation, Lane 4: Enzymes after ion exchange chromatography, Lane 5: Enzyme after gel filtration.

pH optimum and pH stability

The standard phytase assay was performed using a variety of buffers from pH 2.0 to 9.0. The phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 had a single pH optimum at pH 4.5 and was virtually in active below pH 2 or above pH 7.0. The effect on enzyme stability was studied in the range 2.0-9.0 at 4°C. In the pH range from 2.5 to 7 the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 was rather stable, but below pH 2 or above pH 7.5 a rapid declined in activity was observed. Within 10 days, more than 80% residual activity was retained within pH 5.0 - 6.0, but at pH 3, 50 % and at pH 7, 60% of the initial activity was lost. The enzyme shares many enzymatic properties in common with other phytate-degrading enzyme (Irving, 1980; Nayini and Markakis, 1986) but the enzyme shows some differences to phytase from *Enterobacter* sp. 4 (Yoon et al., 1996). The phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 has acidic pH optima (pH 4.5) with rapid drop in activity at pH values above 6 but *Enterobacter* sp. 4 exhibit maximum activity at pH 7-7.5.

Temperature optimum and thermal stability

The temperature profile of purified phytate-degrading enzyme from *Enterobacter sakazakii* ASFA279 was determined from 35 to 90°C using the standard phytase assay. The temperature optimum was found to be 45-55°C. In order to determine thermal stability, the phytate-degrading enzyme was incubated at different temperatures, cooled to 4°C and assayed using the standard phytase assay. The enzyme lost no activity in 90 min at temperature up to 50°C. When exposed for 90 min at 55°C, it retained 87% and at 90°C, 22% of the initial activity. The enzyme has a moderate temperature optimum (45-55°C) that typical for bacterial phytate-degrading enzymes (Greiner, 2004; Greiner et al., 1997, Greiner et al., 1993; Kim et al., 2003; Sajidan et al., 2004; Tambe et al., 1994) but it able to retain its activity (22%) when exposed at 90°C for 15 min. Phytases with high-temperature optima are desirable in animal feed industry because feed pelleting involves a step of 80-85°C for a few seconds.

Substrate selectivity

In order to evaluate the substrate selectivity of the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279, several phosphorylated compounds, in addition to phytate, were used for relative rates calculation and, K_M and V_{max} 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. The results of the hydrolysis are summarized in Table 2. The enzyme showed narrow substrate specificity with the highest affinity for phytate but phytate was not the compound with the highest relative rate of hydrolysis. The highest turnover numbers was found to be GTP. However phytate seems to be the *in vivo* substrate of this enzyme, because k_{cat}/K_M value was highest for this phosphorylayed compound. GTP, pyridoxal-5-phosphate ρ-nitrophenyl phosphate and β-naphthyl phosphate can be categories as fair substrates, whereas ATP and ATP haver to be considered to be poor substrates. All other phosphorylated compounds studied were not hydrolysed by the enzyme. Only a few phytate-degrading enzymes have been described as highly specific for phytate. Similarly, the phytate-degrading enzymes from *Bacillus* sp. DS11 (Kim et al., 1998) and *Pseudomonas* sp. (Irving and Cosgrove, 1971) have no activity on phosphate esters such as AMP and β-glycerophosphate. The K_M of the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 for phytate was determined to be 760µmol/l. This is double to those found for other phytate-degrading enzyme from bacterial sources, i.e. 340 µmol/l for *Pantoea agglomerans* (Greiner, 2004), 300 µmol/l for *Klebsiella terrigena* (Greiner et al., 1997), *Klebsiella pneumoniae* (Sajidan et al., 2004) and 460 µmol/l for *Citrobacter braakii* (Kim et al., 2003). Higher Km means lower affinity. This means that the enzyme not binds the substrate tightly and high concentrations of substrate are needed to saturate the enzyme and reach the maximum catalytic efficiency of the enzyme.

Table 2. Kinetic constants for the hydrolysis of phosphorylated compounds by the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 at temperature 50°C and pH 5.0

Substrate	Relative Activity (%)	K_M *(mM)*	k_{cat} (s^{-1})	k_{cat} / K_M $(s^{-1}M^{-1})$
phytate	100 ± 0.8	0.76 ± 0.05	4.14 ± 0.21	5540
β-naphthyl phosphate	13 ± 1.3	0.89 ± .02	0.28 ± .02	315
ρ-nitrophenyl phosphate	77 ± 1.9	14.15 ± 0.19	9.68 ± 0.03	684
pyridoxal-5-phosphate	69 ± 1.8	6.93 ± 0.25	3.93 ± 0.21	567
ADP	27 ± 1.2	5.23 ± 0.11	0.88 ± 0.08	168
ATP	25 ± 0.8	3.16 ± 0.08	0.40 ± 0.01	127
GTP	201 ± 11.2	3.85 ± 0.10	3.69 ± 0.02	958
α-naphthyl phosphate	0	-	-	-
α-D-glucose-6-phosphate	0	-	-	-
β-glycero phosphate	0	-	-	-
D-fructose-6-phosphate	0	-	-	-
AMP	0	-	-	-
NADP	0	-	-	-

Hydrolysis of Na-phytate was taken as 100%; Temperature: 50°C; buffer: 0.1 M Na-acetate buffer, pH 5.0; enzyme concentration: 2.8 U/ml. Abbreviations: AMP, adenosine-5'- monophosphate; ADP, adenosine-5'-diphosphate; ATP, adenosine-5'-triphosphate; GTP, guanosine-5'- triphospahte; NADP, nicotinamide adenine dinucleotide phosphate.

Effector studies

The study of the effect of metal ions on enzyme activity showed that none of them had an activating effect when used at a concentration between 10^{-4} and $10^{-3}M$. Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Ag^{+}, and Al^{3+} had little or no effect on enzyme activity, while Fe^{3+}, Cu^{2+}, and Zn^{2+} showed strong inhibitory effects. The inhibitory effect may be due to the formation of poorly soluble complexes of the metal ions with phytate, which may decrease the active concentration of phytate in the assay (Wang et al., 1980). When compounds which tend to chelate metal ions such as 1, 10-phenanthroline, EDTA, citrate, or tartrate were tested for their effect on enzyme activity, it was noted that none of them was inhibitory effect at a concentration from 10^{-4} and $10^{-3}M$. But molybdate, vanadate, fluoride and phosphate were found to be strong inhibitor on the phytate-degrading activity of the purified enzyme from *Enterobacter sakazakii* ASUIA279. Phytate-degrading enzymes from different bacteria differed in their requirement for metal ions for their activity. Phytate-degrading enzyme of *Selemonas ruminantium* (Yanke, 1999) was strongly inhibited by in the reaction mixtures containing 5 *mM* Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+}. The partially purified enzyme from *Klebsiella oxytoca* MO-3 was strongly inhibited by NaF, $Zn^{2+}Fe^{2+}$, and Cu^{2+} but it was not inhibited by EDTA or N-ethlmaleimide (Jareonkitmongkol et al., 1997). Further work is in progress to elucidate the structure of the phytate-degrading enzyme and to answer the questions on the catalytic mechanism of the phytate-degrading enzyme from *Enterobacter sakazakii* ASUIA279 and on the final product of enzymatic phytate degradation.

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