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CHARACTERIZATION OF PARTIALLY PURIFIED PHYTASE FROM RICE BRAN

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ABSTRACT

The crude extract of phytase from rice bran was prepared by the extraction with 2.0% $CaCl_2$ solution and partially purified by using of ammonium sulphate (35-80% saturation) followed by the dialysis in distilled water. The specific activity of enzyme was increased by 7.4 and 10.8 folds after ammonium sulphate fractionation and dialysis, respectively comparing with the crude extract.

Characteristics of the partially purified rice bran phytase was also studied. The enzyme showed an optimum pH of 5.0 and optimum temperature of 50°C. Data concerning the rate of thermal inactivation revealed that the enzyme retained about 80% and 30% of its activity when heated for 10 min. at 60 and 70°C, respectively. The enzyme activity was reduced at high substrate concentrations with an optimum substrate concentration of 3.0 mM sodium phytate. The K_m value was found to be $1.66x10^{-3}$ M and the V_{max} was 0.95 µM Pi liberated/min/ml enzyme.

The enzyme activity was markedly influenced by the presence of some metal salts. Each of $CoCl_2$, $MnSo_4$, $CaCl_2$ and $MgSO_4$ showed an activating effect on the enzyme reaction when added at concentrations of $1x10^{-4}$ or $1x10^{-3}$ M. However, both $ZnSO_4$ and $NiCl_2$ caused partial inhibitory effect, while $FeSO_4$ and $CuSO_4$ were found to strongly inhibit the enzyme activity.

Keywords: myo-inositol phosphate; phytase; phytic acid; rice bran.

INTRODUCTION

Phytase (myo-inositol hexaphosphate phosphohydrolase, EC 3.1.3.8) catalyzes the hydrolysis of phytic acid [myo-inositol (1,2,3,4,5,6) hexakisphosphate], the major storage form of phosphate in seeds and pollen, to a series of lower phosphate esters of myo-inositol and phosphoric acid (Reddy *et al*., 1982). Phytate, which forms insoluble complexes with nutritionally important metals such as zinc, calcium, magnesium and iron, reduces the bioavailability of these compounds (Erdman and Poneros-Schneier, 1989; Fox and Tao, 1989). Therefore, phytate is regarded as an antinutritive factor. As monogastric animals including man are lacking in phytases, phytate does not degraded during digestion in the small intestine; hence the phytate phosphate is unavailable. Therefore, phytases are of interest for biotechnical applications, especially for reducing food phytate (Simons *et al*., 1990). Addition of phytase to food might diminish antinutritional effects of food due to high phytate content. Phytases are present in plants, certain animal tissues and some species of microorganisms (Irving, 1980 and Wang *et al*., 1980). In food for human

consumption, plant phytase has two advantages over phytases from microorganisms: higher acceptance of the food by consumers and a lower risk of allergic reactions (Konietzny *et al*., 1995). Phytase activity has been studied in wheat, barley, tritical, canola, sunflower, broad beans, spelt, lupin and germinated oat (Peers, 1953; Preece and Gray, 1962; Singh and Sedeh, 1979; Houde *et al*., 1990; Rizk, 1991; Konietzny *et al*., 1995; Silva and Trugo, 1996 and Greiner and Alminger, 1999, respectively). On the other hand, less attention has been paid to phytase from rice bran. In Egypt, the annual production of rice amounts to about 4.3 million tons. The bran which is an important by-product obtained during rice milling amounts to 1/10 of the weight of rice grain. It is rich in protein, oil, vitamins and minerals (Soheir, 1996). Yoshida *et al*., 1975 found that dephosphorylation of phytic acid occurred in the aleurone particles of rice grain and the active enzyme was also associated with aleurone particles; i.e. within the same location.

One approach to increasing the utilization of agricultural products is fractionation of low-value materials to yield high-value products. This study was aimed to investigate rice bran as a source of phytase. Isolation and characterization of the enzyme were carried out.

MATERIALS AND METHODS

Materials:

Fresh rice bran samples of the “Sakha 2105” variety was obtained from a rice mill at Beheira Governorate, Egypt. Samples were ground to a 80 mesh size and kept at 4±1°C. Phytic acid in the form of sodium phytate was purchased from Sigma Chemicals Co. St Louis, USA. All chemicals were AR grade.

Preparation of enzyme extract:

The crude enzyme extract was prepared and partially purified according the method described by Lolas and Markakis (1977) which was adapted by Rizk (1991) as follows: Finely ground rice bran (80 mesh) was extracted with a 10:1 (v/w) ratio of 2% $CaCl_2$ in distilled water to rice bran in a mechanical shaker, 3000 rpm for one hour on an ice bath and centrifuged (5000 xg) at 2^{o}C for 30 min. The supernatant was filtered through cheese cloth to exclude the floating fraction. The clear solution had a pH of 5.0 and used as crude enzyme.

Partial purification of the crude enzyme:

Ammonium sulphate was added to the crude enzyme solution with a continous mechanical stirring until 35% saturation was obtained as calculated by using a nomogram for $(NH_4)_2SO_4$ solution (Dixon *et al*., 1979). The mixture was kept for 30 min at 2°C and centrifuged at 5000 xg for 20 min at 2°C. The residue was dissolved in a small volume of 0.1 M acetate buffer pH 5.6 whereas, the supernatant was made to 80% $(NH_4)_2SO_4$ saturation; chilling and centrifuging were followed as previously. The fraction precipitated between 35% and 80% $(NH_4)_2SO_4$ saturation, contains most of

the phytase activity, was dissolved in a small volume of 0.1 M acetate buffer, pH 5.6 and dialyzed for 48 hours in distilled water. This partially purified phytase was used as the enzyme source for the experiments carried out in this investigation.

Determination of phytase activity:

Phytase activity was determined by measuring the inorganic phosphorus (Pi) liberated by the action of the enzyme as described by Houde *et al*. (1990). The assay involved the incubation (30 min.) in a water bath (50°C) of test tube containing 0.2 ml enzyme extract and 3.8 ml sodium acetate buffer (0.1 M, pH 5.0). The reaction was initiated by adding sodium phytate solution (2 mM, 0.1 ml) and halted by addition of 2 ml trichloroacetic acid 20%. The activity values were corrected from a control which contained boiled enzyme under the same conditions. Inorganic phosphate (Pi) was determined according the method of Chen *et al*. (1956); Phytase activity was expressed as International Units (IU) of phytase; one IU is defined as the amount of enzyme which liberates one µmol of Pi/min. under the conditions of the assay.

Protein determination:

Protein content was determined according to the method of Schacterle and Pollack (1973) using bovine serum albumin as standard.

Enzyme characterization:

Effect of pH on phytase activity:

The effect of pH (mixtures adjusted to 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4 and 5.6) on phytase activity was determined in 0.1 M acetate buffer. Incubation was carried out at 50°C for 30 min.

Effect of temperature:

Phytase assay mixtures were incubated at pH 5.0 for 30 min. over the temperature range 35-70°C at 5°C intervals to determine the optimum temperature for enzyme activity.

Effect of substrate concentration:

Optimum substrate concentration for phytase activity was determined by incubation of 0.2 ml enzyme solution for 30 min. at 50°C in 0.1 M acetate buffer pH 5.0 with sodium phytate at concentrations from 0.5-8.0 mM. The K_m and V_{max} values were determined by the classical method of Lineweaver and Burk (1934).

Thermal inactivation of phytase:

Enzyme in 0.1 M acetate buffer, pH 5 was heated for 10 min. at temperature ranging from 30-80°C in a water bath, cooled, sodium phytate was then added at concentration of 3 mM. Phytase activity was assayed after incubation for 30 min at 50°C.

Effect of incubation period:

Phytase assay was performed in 0.1 M acetate buffer pH 5 at 50°C for incubation period from 0.0 to 3.0 hours.

Effect of metal salts:

Metal salts ($MgSO_4$, $ZnSO_4$, $MnSo_4$, $CuSo_4$, $FeSo_4$, $CaCl_2$, $AlCl_3$, $CoCl_2$ and $NiCl_2$) were tested at concentrations of 10^{-3} M, 10^{-4} M and 10^{-5} M to find out the effect of these metal salts on phytase activity.

RESULTS AND DISCUSSION

Partial purification of phytase:

For studying the properties and behaviour of an enzyme, it is necessary to obtain the enzyme as pure as possible. The summary of extraction and partial purification procedures of rice bran phytase is shown in Table (1). The total activity of the crude extract which prepared by using of 2% $CaCl_2$ solution was 1.76 units and the corresponding specific activity was 0.54 unit/mg protein. According to Lolas and Markakis (1977), the extraction of the enzyme from bean flour with water or buffers and subsequent ammonium sulphate fractionation could not avoid contamination of the enzyme by the natural substrate, phytic acid, present in the bean flour. While a 2% $CaCl_2$ solution was found satisfactory in separating the enzyme from phytic acid. Besides, Nayini and Markakis (1984) reported that crude phytase extracted from yeast macerate by mixing nine volumes of a 2% $CaCl_2$ solution with one volume of yeast macerate resulted in an extract of higher phytase activity than extracts prepared with distilled water, solution of NaCl, KCl, 0.1 M tris-maleate buffer and 0.2 M acetate buffer.

The salt employed in partial purification of rice bran phytase is ammonium sulfate on account of its high solubility and the absence of harmful effects on most enzymes. It has in fact a stabilizing action on many enzymes
and it is usually not necessary to carry out the fractionation at a low temperature (Dixon *et al*., 1979).

Table (1): Summary of extraction and partial purification procedures of rice bran phytase.

Fraction (F)	Phytase activity (I.U.)*	Protein (mg/ml)	Specific activity (U/mg protein)
1- Crude extract (2% $CaCl_2$). (FI)	1.76	3.24	0.54
2- First $(NH_4)_2SO_4$:			
Fraction-I brought to 35% saturation with $(NH_4)_2SO_4$ and centrifuge. (FII).			
(a)- Supernatant	2.35	1.70	1.38
(b)- Preciptate	0.25	1.45	0.17
3- Second $(NH_4)_2SO_4$:			
Fraction-II(a) brought to 80% saturation with $(NH_4)_2SO_4$ and centrifuge (FIII).			
(a)- Supernatant	0.20	1.05	0.19
(b)- Preciptate	3.40	0.85	4.00

4- Partially purified enzyme: Fraction III (b) dissolved in sod. acetate buffer and then dialyzed (FIV)	4.65	0.80	5.81

***(IU): International unit; one IU is defined as the amount of enzyme which liberates one µmol of Pi/min. under the conditions of assay.**

Data in Table (1) indicated that, the purification with ammonium sulfate followed by dialysis led to a noticeable increase in total activity, specific activity and a remarkable reduction in protein content for phytase extract. The specific activity after fractionation with 35% and 80% saturation of ammonium sulfate solutions increased to about 2.5 and 7.4 folds than the crude extract, respectively. Further increment in the specific activity was attained after dialysis reaching to 10.8 folds in relation to the crude extract. The increase in enzyme activity may be due to the removal of naturally occurring inhibitors present in the crude extract as mentioned by Klein (1976).

Effect of pH on phytase activity:

The optimum pH for rice bran phytase activity was found to be 5.0 with a rapid diminution in activity on either side of this optimum (Fig. 1). Various pH values have been reported in the literature as optimum for phytase; 5.3 for navy beans phytase (Lolas and Markakis, 1977); 4.8 for soybean phytase (Sutardi and Buckle, 1986); 5.2 for canola seed phytase (Houde *et al*., 1990); 6.0 for spelt phytase (Konietzny *et al*., 1995) and 5.0 for germinated oat phytase (Greiner and Alminger, 1999).

Effect of incubation temperature:

The optimum temperature for rice bran phytase was about 50°C (Fig. 2). At temperature higher than 55°C, the activity was significantly reduced and at 70°C the enzyme was completely inactive. The relatively high temperature for optimum activity is, presumably, partly a reflection of its thermostability. these results are in agreement with values reported for other plant phytases; 50°C for navy beans phytase (Lolas and Markakis, 1977); 50°C and 55°C also for maize phytase (Chang, 1967 and Laboure *et al*., 1993, respectively). In contrast, a lower temperature optimum (38°C) was reported for germinated oat phytase (Greiner and Alminger, 1999).

Thermal stability of rice bran phytase:

Standard assay mixtures not containing the substrate were maintained for 10 min. at different temperatures from 30 to 80°C at 5°C intervals in a water bath. After exactly 10 min. heating, they were put immediately in an ice bath, substrate was added and the phytase activity was measured according to the standard procedure. The results were compared with that of a control without heat treatment and plotted as percent remaining activity against the temperature of 10 min. heat treatment (Fig. 3). Evidently heating up to 50°C for 10 min. did not reduce the enzyme activity. At 60 and 70°C, the activity decreased to about 80 and 30% of the control value, respectively. The enzyme become completely inactive when heated to 80°C for 10 min. This experiment showed the enzyme to be relatively stable.

Similar results were obtained by Lolas and Markakis (1977) with navy beans phytase.

Effect of incubation time:

Progress curve of the hydrolysis of phytate (time vs velocity) using standard assay mixture at 50°C is presented in Fig. (4). The activity was linearly related to the time of incubation up to 2.0 hours.

Effect of substrate concentration:

Phytase activity increased with increasing the substrate concentration up to 3.0 mM, but was inhibited at concentrations higher than 4.0 mM as shown in Fig. (5). Konietzny *et al*. (1995) reported that phytase activity was inhibited at concentrations of phytate higher than 5.0 mM. This decrease in the activity is probably due to the inhibitory effect of inorganic phosphate liberated during the reaction. Inorganic phosphate was found to inhibit both phytase and phosphatase activity (Mandal *et al*., 1972). Also, Gibbins and Norris (1963) suggested that the inhibition of phytase by high substrate concentrations was indicative of a two-point attachment of phytate to phytase.

From a plot of 1/v against 1/[s] (Lineweaver and Burk, 1934), the Michaelis constant (K_m) was found to be 1.66 x 10^{-3} M and the V_{max} was 0.95 µmol phosphorus liberated/min/ml enzyme (Fig. 6). Great variation in apparent K_m values of phytases from different origins is found in the literature. Values range from 0.091 mM for corn (Chang, 1967); 0.57 mM for wheat bran (Nagia and Funahashi, 1962); 0.15 mM, 0.65 mM and 0.018 mM for beans (Gibbins and Norris, 1963; Mandal *et al*., 1972 and Lolas and Markakis, 1977, respectively) to 2.4 x 10^{-3} M for soybean (Sutardi and Buckle, 1986). This variation might be due to the differences in purity of the enzyme preparations studied and in the experimental conditions (Wang *et al*., 1980 and Silva and Trugo, 1996).

Effect of metal salts:

The effect of various metal salts on partially purified phytase activity was studied and the results are shown in Table (2). Evidently, $MgSO_4$, $MnSO_4$, $CaCl_2$ and $CoCl_2$ activated the enzyme reaction. The activating effect of these metal salts was increased with increasing their concentrations. However, at 1.0 mM concentration, $MgSO_4$ did not retain its activating ability. The activating effects of Mg, Mn, Ca and Co salts have been observed earlier by Lolas and Markakis (1977) for navy bean phytase and by Houde *et al*. (1990) for canola seed phytase.

On the other hand, $ZnSO_4$ and $NiCl_2$ partially inhibited the phytase activity while $FeSO_4$ and $CuSO_4$ were found to strongly inhibit the enzyme reaction at all the studied concentrations. These results are similar to that reported by Konietzny *et al*. (1995) for spelt phytase. Rizk (1991) postulated that these metal ions have a strong affinity for phytic acid and compete with the enzyme for the substrate. Also, Greiner *et al*. (1998) attributed the reduced phytase activity in the presence of Fe^{+2} and Fe^{+3} to a lower phytate concentration because of the appearance of Fe-phytate precipitate.

Table (2): The effect of metal salts on rice bran phytase activity.

Metal salt	Relative activity[a]		
	10^{-5} M	10^{-4} M	10^{-3} M
$MgSO_4$	105	115	100
$ZnSO_4$	98	80	59
$MnSO_4$	102	105	119
$CuSO_4$	78	44	20[b]
$FeSO_4$	66	35[b]	12[b]
$CaCl_2$	100	100	112
$AlCl_3$	100	98	100
$CoCl_2$	110	116	135
$NiCl_2$	96	90	64

a: The activity without added metal salt was taken as 100%.
b: Precipitation was observed upon addition of substrate.

In conclusion, this study has shown that rice bran could be a source of phytase enzyme. The role of this enzyme as a natural agent for reducing phytate could be extended for potential applications in plant foods and plant-derived food products. Further experiments in this respect are under taken.

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خصائص انزيم الفيتيز المنقى جزئياً من رجيع الكون

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اشتملت الدراسة على استخلاص انزيم الفيتيز من رجيع الكون باستخدام كلوريد الكالسيوم 2% ثم اجراء التنقية الجزئية بواسطة كبريتات الأمونيوم (35 ، 80% تشبع) ويعقبها الانتشار الغشائى فى الماء المقطر . وقد إزداد النشاط النوعى للانزيم بمقدار 4ر7 ، 8ر10 مرة عقب إجراء كل من التنقية بكبريتات الأمونيوم والانتشار الغشائى على التوالى وذلك مقارنة بالمستخلص الخام .

كذلك تم دراسة خصائص الانزيم المتحصل عليه بعد عمليات التنقية الجزئية وأظهرت النتائج ما يلى :

- كانت درجة الـ pH المثلى لنشاط الانزيم هى 0ر5 ودرجة الحرارة المثلى هى 0ر50°م ، وقد احتفظ الانزيم بحوالى 80 ، 30% من نشاطه عند تعريضه لمدة 10 دقائق لدرجات حرارة 60 ، 70°م على التوالى .
- ازداد النشاط الانزيمى بزيادة تركيز مادة التفاعل حتى وصل الى أقصاه عند تركيز 0ر3 ملى مول من فيتات الصوديوم وكانت قيمة كل من K_m , V_{max} 66ر1×10^{-3} مول ، 95ر0 ميكرومول فوسفور / دقيقة /مل انزيم على التوالى .
- أوضحت النتائج أيضا أن نشاط انزيم الفيتيز يتأثر تأثراً ملحوظاً بوجود الأملاح المعدنية حيث أظهرت أملاح كبريتات المنجنيز وكبريتات الماغنسيوم وكلوريد الكوبالت وكلوريد الكالسيوم تأثيراً منشطاً عند تواجدها بتركيزات 10^{-4} ، 10^{-3} مول بينما انخفض النشاط جزئياً فى وجود أملاح كبريتات الزنك وكلوريد النيكل أما أملاح كبريتات الحديدوز وكبريتات النحاس فقد أدت الى حدوث تثبيطاً واضحاً لنشاط الانزيم .