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Effect of germination temperature on characteristics of phytase production from barley

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Abstract

The effects of germination temperature on the growth of barley seedlings for phytase production were studied at 15, 20 and 25 °C for 6–10 days. The growth rate of the barley seedlings was increased as the germination temperature was increased. The initial rate of total protein production was closely coupled to that of the barley growth, and the rate of total protein production tended to increase as the germination temperature was increased. SDS–PAGE analysis of total protein from the barley seedlings showed time-dependent appearance and disappearance of protein bands. Although no significant phytase activity was detected at zero time of germination, a significant increase in phytase activity up to 7.9-fold occurred during the first several days of germination then decreased. Phosphate production (viz. phytate degradation) in the barley seedlings occurred rapidly at the beginning of germination. However, the rate of production continued to decrease with further germination. A time lag of about 1–2 days between the rate of total protein production and that of phytase production was observed. Unlike the extent of total protein production, that of phytase production was similar irrespective of germination temperature. Partial purification of a crude enzyme extract by hydrophobic interaction chromatography resulted in two phytase fractions (PI and PII). Zymogram analysis demonstrated that PI had two bands with molecular masses of about 66 and 123 kDa while PII had one band corresponding to a molecular mass of about 96 kDa. The optimal temperature for PI was found to be 55 °C, while it was 50 °C for PII. The enzyme fraction PI had a pH optimum at 6.0, whereas the optimum pH for PII was found to be 5.0. Addition of 0.1% (v/v) Tween 80 was found to increase enzyme activity significantly (i.e., 167% for PI and 137% for PII). Phytate in cereals including barley, rice, corn and soybean degraded effectively by the treatment of the barley phytases.

Keywords: Barley; Germination; Phytase; Phytate degradation; Zymogram

1. Introduction

Phytate (*myo*-inositol-(1,2,3,4,5,6)hexaphosphate), an antinutrient factor in animal feeds, is regarded as the primary storage form of both phosphate and inositol in plant seeds and grains (Greiner et al., 1998; Bergman et al., 2000). It has been reported that phytate content ranges from 0.5% to 5.0% (w/w) in cereals and legumes (Laboure et al., 1993; Greiner et al., 2000). The stepwise hydrolysis of phytate to phosphate and inositol occurs by the action of phytase. Phosphatase hydrolizes a broad spectrum of phosphate esters, while phytase is a phytate-specific phosphatase (Greiner et al., 1998). The phosphatase is not capable of degrading phytate (Konietzny et al., 1995; Greiner et al., 2000). Two types of phytase have been identified which initiate the

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hydrolysis of phytate at either the 3- or 6-position of the inositol ring (Konietzny et al., 1995; Greiner et al., 2001). The phytases from microorganism such as *Aspergillus niger* is considered as 3-phytases (EC 3.1.3.8), while plant phytases as 6-phytases (EC 3.1.3.26) (Turk et al., 1996; Greiner et al., 1998, 2000). The plant phytases preferentially hydrolize the phosphoester bond at 6-position of the *myo*-inositol residue. There are enormous potential for application of phytase in the animal feed industry (Gautam et al., 2002; Casey and Walsh, 2003; Martin et al., 2003).

The principal function of phytase in seeds or grains is to produce inorganic phosphate from phytate during germination. The inorganic phosphate thus produced is then utilized for the purpose of plant growth. Previous authors have studied various plant phytases from maize, barley, spelt, canola seed and rye (Houde et al., 1990; Laboure et al., 1993; Konietzny et al., 1995; Greiner et al., 1998, 2000). It appears that phytase activity usually increases on germination and the types of phytase may be plant-dependent. Although many investigators have tried to purify phytases from many germinating seeds, only a few phytases have been purified to homogeneity or near homogeneity (Laboure et al., 1993; Konietzny et al., 1995). Unlike the phytases obtained from microorganisms, it was very difficult to purify plant phytases from contaminating nonspecific phosphatases. It was reported that the phytase activity was generally unstable and the specific activity of purified phytase varied from one experiment to another (Laboure et al., 1993).

Although significant increases in phytase activity in various germinating seedlings have already been reported, to our knowledge, little information is available concerning the effects of germination temperature on the growth of seedlings for phytase production (Rimsten et al., 2002). In the present investigation, the effects of temperature on the pattern and extent of phytase production as well as those of total protein production from the germinating barley were studied. After partial purification of a crude enzyme extract, molecular masses of barley phytases were estimated from a zymogram analysis. Properties of the isolated phytases in terms of temperature, pH and supplementation of surfactant were also studied.

2. Methods

2.1. Seed germination

Barley seeds were obtained from a local market. The procedure of germination was similar to that reported previously (Konietzny et al., 1995; Greiner et al., 1998). The seeds were soaked in 0.1% Tween 80 for 5 min and then in 0.75% H_2O_2 for 1 min. After soaking, the barley seeds were thoroughly rinsed with sterile water and then soaked again in distilled water for 16 h at 4 °C. The pretreated barley seeds were strained through two layers of gauze and then allowed to germinate in the dark at 15, 20 and 25 °C for 6–10 days. The seeds were rinsed once a day with sterile water during the period of germination.

2.2. Preparation of enzyme extract

Barley seedlings, resulting from the germination, were dried at room temperature and then were ground in a kitchen blender. One gram (dry matter basis) of the ground seedlings was mixed with a 10 ml of 0.1 M acetate buffer (pH 5.0). After shaking at 4 °C for 16 h, the solid debris was removed by centrifugation at 8000*g* for 30 min and the supernatant was analyzed for phytase activity and phosphate content.

2.3. Estimation of phytase and phosphate

Phytase activity was determined by measuring the release of phosphate from sodium phytate (Houde et al., 1990). One unit (1 U) of phytase activity was defined as 1 μmol phosphate released per min under the following condition: pH 5.0, temperature 37 °C , and reaction mixture consisting of 600 μl of 0.2% (w/v) sodium phytate (Sigma) in 0.1 M acetate buffer (pH 5.0) and 150 μl of enzyme sample. The enzyme reaction stopped by adding 750 μl of 5% (w/v) trichloroacetic acid after incubation for 30 min. The liberated phosphate was determined using the method as reported previously (Fiske and Subbarow, 1925).

2.4. Protein determination

Total protein concentration of the supernatant was determined according to the method described by Bradford (1976), with bovine serum albumin as a standard.

2.5. Gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% gels as described by Laemmli (1970). Proteins were stained by Coomassie Brilliant blue R-250.

2.6. Partial purification of phytase by hydrophobic interaction chromatography

The crude enzyme extract obtained from the 5-day germinated seedlings at 20 °C as a source for phytase isolation was treated to 70% ammonium sulfate saturation. The precipitate was collected by centrifugation at 8000*g* for 20 min and suspended in 50 mM acetate buffer (pH 5.0) and then dialyzed against the same buffer with 1 M ammonium sulfate. The dialyzed ammonium sulfate