Properties of a highly specific phytate-degrading enzyme with an acid pH optimum from a bacterium isolated from Malaysian waste water

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The stepwise release of phosphate from phytate [*myo*-inositol (1,2,3,4,5,6) hexa*kis*phosphate], the major storage form of phosphate in plant seeds and pollen, is initiated by a class of enzymes collectively termed phytases. Since an ideal phytase for all application does not exist, there is still interest in an enzyme more suitable for the intended use. In this report we present the properties of a phytase purified from a bacterium isolated from Malaysian waste water, which might find application as an animal feed supplement.

In the bacterial cells a significant phytate-degrading activity was detectable during all stages of growth and the activity did not increase significantly after the cells reached the stationary phase. Therefore, this phytase is a constitutive enzyme and, in contrast to other bacteria, its synthesis is not triggered by nutrient or energy limitation (Konietzny and Greiner, 2004). No phytate-degrading activity was detectable in the dialyzed culture medium of growing bacterial cells. As no inhibitors were present in the culture medium there was no question of an extracellular phytase. During an osmotic shock procedure more than 90% of the phytate-degrading activity obtained after lysis of the bacterial cells was released. Therefore, the phytase must be regarded as a periplasmatic enzyme.

The phytase was purified about 180-fold to apparent homogeneity by ion-exchange chromatography and gel-filtration with a recovery of 10% of the phytate-degrading activity in the crude extract. The enzyme had an activity of about 1106 U mg^{-1}. Gel filtration of the native enzyme on a calibrated Sephacryl S-200 column gave a molecular mass of 42.000 ± 1.500 Da, with elution position being measured by determination of enzyme activity. Lower molecular mass species or higher molecular mass aggregates were not observed. The phytase appeared homogeneous by polyacrylamide gel electrophoresis under non-denaturing conditions at pH 8.3 and pH 4.8 and gave a single protein band upon SDS gel electrophoresis after Coomassie staining of the gels. These results indicate 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 41.500 ± 2.500 Da. The enzyme is therefore a monomeric protein.

The standard phytase assay was performed using a buffers from pH 1.0 to 9.0. The purified enzyme had a single pH optimum at pH 4.5 and was virtually inactive below pH 7.0. At pH 3.0 only 40% of the activity at optimal pH was observed, while at pH 2.5 this value was 20%. Within 14 days the phytase did not lose activity between pH 3.0 to 8.0, but at pH values below 2.0 a rapid decline in activity was observed. At pH 1.5, 72% and at pH 9.0, 65% of the initial activity was lost during 24 h. The temperature optimum was 65°C. The apparent activation energy was estimated at pH 4.5 from the slope of log V_{max} versus 1/T. The data showed excellent linearity from 15 to 65°C. The Arrhenius activation energy for the hydrolysis of phytate was calculated to be 37.5 kJ/mol. To check thermal stability, the purified enzyme was incubated at different temperatures, cooled to 4°C and assayed using the standard phytase assay. The enzyme

lost no activity in 10 minutes at temperatures up to 65°C. When exposed for 10 minutes at 70°C it retained 50% of the initial activity, while at 80°C it retained 12%.

To determine the substrate selectivity of the purified phytase, several phosphorylated compounds (GTP, ATP, ADP, AMP, NADP, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, 1-naphthylphosphate, 2-naphthylphosphate, pyrophosphate, phosphoserine, 2-glycerophosphate, pyridoxalphosphate), in addition to phytate, were used for K_m and V_{max} estimation by detecting the release of the phosphate ion during hydrolysis using formation of a soluble phosphomolybdate complex in an acidic water-acetone mixture. Only phytate was identified as a substrate. All other compounds tested were not significantly hydrolyzed by the purified enzyme. The kinetic parameters for the hydrolysis of phytate were determined to be K_m = 0.15 mmol L^{-1} and k_{cat} = 1164 sec^{-1} at pH 4.5 and 37°C.

Like other bacterial phytate-degrading enzymes, the purified enzyme showed a substrate inhibition (Konietzny and Greiner, 2002). The activity of the purified enzyme was inhibited at substrate concentrations > 7 mM. 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+}, Co^{2+}, Ag^{+}, Hg^{2+}, Cu^{2+} had little or no effect on enzyme activity, while Zn^{2+}, Fe^{2+}, and Fe^{3+} showed strong inhibitory effects. The reduced phytate-degrading activity in the presence of Fe^{2+} and Fe^{3+} is attributed to a lower phytate concentration in the enzyme assay because of the appearance of a Fe-phytate precipitate. When compounds which tend to chelate metal ions, such as *o*-phenanthroline, EDTA, oxalate, citrate or tartrate, were tested for their effect on enzyme activity, none were inhibitory at concentrations between 10^{-4} to 10^{-3} M. Fluoride, a known inhibitor of different phytate-degrading enzymes from bacteria and the hydrolysis product phosphate as well as its structural analogous molybdate, wolframate and vanadate were strong inhibitors of the purified enzyme. Fluoride inhibited the hydrolysis of phytate with a K_i value of 112 μmol L^{-1}.

Phytate-degrading enzymes used as feed additives should be effective in hydrolyzing phytate in the digestive tract, stable to resist inactivation by heat from feed processing and storage, and cheap to produce. The purified phytase shares many enzymatic properties in common with other histidine acid phytases. However, some properties make the enzyme attractive for an application as a feed supplement. Compared to the commercially available phytases from *Aspergillus niger* and *Peniophora lycii*, the purified enzyme exhibit a higher thermal stability (Ullah and Sethumadhavan, 2003). Like the phytase of *A. niger*, but in contrast to the *P. lycii* enzyme, the purified phytase showed considerable activity below pH 3.0. In addition, the purified phytase is more resistant to pepsin and pancreatin than the commercially available *A. niger* phytase. Its protease resistance is comparable with that of the phytase from *Escherichia coli*. The enzyme is specific for phytate and dephosphorylates phytate at least to *myo*-inositol bisphosphate. The high affinity for phytate and the high specific activity are also desirable for the intended application. The K_m values are comparable to those of the phytases from *A. niger* and *P. lyccii* and the specific activity is in the same magnitude as the specific activity of the phytase from *P. lycii*.

References

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