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A New *in Vitro* Enzymatic Method to Evaluate the Protective Effect of Phytic Acid Against Copper Ions

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Abstract: Copper (Cu^{2+}), a component of the mitochondrial electron transport chain which also functions in iron absorption, mobilization and in maintenance of brain neurotransmitter levels, is well known to generate cytotoxic reactive oxygen species (ROS). The aim of this study was to test a sensitive, simple and low cost enzymatic method based on the urease inhibition by very low concentrations of copper and to use this technique to study the protective effect of phytic acid against copper ions. In this way, an enzymatic method based on the ability of urease to be inhibited by very low concentrations of copper ion was utilized. Inhibition curves showed that copper (0.79 and 1.57 μ M) promoted a 43.82 ± 2.72 and 71.84 ± 6.29% inhibition of the enzyme activity, respectively. Enzyme inhibition by the same copper concentrations in the presence of phytic acid (10 mM) were markedly lower. In conclusion, phytic acid has a copper chellating capacity that may be useful in preventing copper interaction with biomolecules and, accordingly, in lowering the generation of ROS.

Key word: Urease, copper, ions, phytic acid

Introduction

Phytic acid (CAS 83-86-3) is recognized as the storage form of phosphorus in many plants, particularly in cereals, nuts, oil seeds, legumes, pollen and spores (Raboy, 2001). Containing about 28% phosphorus, in the form of phosphoric acid, phytic acid has been used as an antioxidant and could conceivably be a protective agent in the human diet. Phytic acid forms salts with minerals or complexes with proteins called phytates and potently and effectively prevents pathological calcifications (Midorikawa *et al*., 2001; Shears, 2001). In nuclear medicine, technetium 99m labeled-phytic acid is used to perform hepatic scintigraphy (Saha, 1998; Gomes *et al*., 2002). Phytic acid is also a chelator of divalent and trivalent cations (Grases *et al*., 2001). A maximum of six phosphate groups of the inositol ring can complex cations such as calcium, magnesium, iron, zinc and copper. Being a highly negatively-charged molecule at a wide range of pH, it can also form insoluble complexes with other cations (Raboy, 2001; Gersonde and Weiner, 1982; Rimbach and Pallauf, 1998).

Copper is stored in some organs such as the liver and is required for the activity of enzymes associated with iron metabolism, elastin and collagen formation, melanin production, integrity of central nervous system and normal red blood cell formation (Kumar, 2002). The essential problem with metals is that they are important for many industries and sometimes may reach high concentrations in the environment (air, water, food and soil), promoting adverse effects to life. Current enzymatic methods for metal detection are largely based on the high affinity between metals and sulphydril (-SH), amine or carboxyl groups. Some of the available methods for determination of metal content in environmental samples are based on enzyme inhibitory properties of metals. Calorimetric, electrochemical and ion-selective potentiometry measurements are also used to quantify the effect of metals or its presence in different samples (Mealor and Townshend, 1968; Winquist *et al*., 1988; Bertocchi *et al*., 1999; Zaborska *et al*., 2001; Champagne, 1987; Krawczyk *et al*., 2000). In this study, we have tested a sensitive, simple and low cost enzymatic method based on the urease inhibition by very low concentrations of copper and used this technique to study the protective effect of phytic acid against copper

Table 1: Effect of phytic acid on urease inhibition by the copper ion

Copper	% Inhibition of Urease Activity		
[μM]	No Phytic Acid	10 mM Phytic Acid	20 mM Phytic Acid
None	0.00	2.96 ± 0.56	17.52 ± 3.37
0.79	43.82 ± 2.72	20.55 ± 1.49*	15.05 ± 2.77*
1.57	71.84 ± 6.29	35.48 ± 5.83*	28.65 ± 1.07*

Copper and phytic acid were added to glycerol phosphate buffer before the addition of urease. After a 15 min pre-incubation, the enzymatic reaction was started. Experimental details in the text. Results (in % of enzyme inhibition) are means ± S.D of ten experiments.* Phytic acid reduces significantly the urease inhibition by copper ($p<0.01$)

ions.

Urease, a nickel-dependent metallo-enzyme, catalyses urea hydrolysis, forming ammonia (NH_3) and carbon dioxide. The primary role of this enzyme is to allow the organism to use externally or internally generated urea as a nitrogen source (Jabri *et al.*, 1995; Srivastava and Kayastha, 2000). The urea analysis is of considerable interest in clinical chemistry, food chemistry and environmental monitoring (Pizzariello *et al.*, 2001; Melo *et al.*, 2003). Tests using urease to detect metals in water confirmed some specificity to copper, silver, mercury and tin (Winquist *et al.*, 1988; Bertocchi *et al.*, 1999; Zaborskaet *et al.*, 2001; Champagne, 1987; Krawczyk *et al.*, 2000). Krawczyk *et al.* (2000) have reported urease inhibition for copper (Cu^{2+}) ions about 10 times lower effect than for silver (Ag^{+}) ions and, moreover, for 0.1 μM Cu^{2+} practically no inhibition effect was observed while for 1 μM this effect was 5-folder lower than for mercury (Hg^{2+}) ions. Juszkiewicz *et al.* (2004) demonstrated that the inhibition of urease by garlic extract should be attributed to the reaction of thiosulfinates with the SH-group found in the active site of urease. Traditional methods for urea determination generally involve the formation of a colored complex either with urea itself or with the ammonia obtained by urea decomposition (Melo *et al.*, 2003; Juszkiewiz *et al.*, 2004; Eggenstein *et al.*, 1999). In this communication we used a simple potentiometric procedure to assay the NH_3 produced by urease activity.

Materials and Methods

Copper (Cu^{2+}) atomic absorption standard aqueous solution (copper nitrate, 1 mg/mL, Merck, Germany) and phytic acid ($C_6H_{18}O_{24}P_6$) were purchased from Sigma Chemical Co. (MO, USA). We used a commercial urease preparation (from jack beans, type III - Sigma U-1875) and all other reagents were of analytical grade or better. For urease assay, we used a pre-incubation mixture (final volume 3.0 ml) containing: 1.6 units of urease, and 250 mM phosphate buffer (pH 7.5) in 3% glycerol (control samples). In test samples, this mixture also contained Cu^{2+} (in final incubation concentrations of 0.79 and 1.57 μM) and/or phytic acid (final incubation concentrations of 10 and 20 mM). Corresponding blank samples did not contain urease. After a 15 min pre-incubation at room temperature, incubation was started by pouring urea (100 μl of a 1.67 M solution) at room temperature. The reaction was stopped 30 min latter, by adding NaOH (150 μl, 5.0 M), and NH_4^+ was then measured immediately using a selective NH_4^+ electrode (Orion Research) coupled to a potentiometer. Firstly, the electrode was previously calibrated with two ammonia standards (P_{10} and P_{100}), where 10 μl (P_{10}) and 100 μl (P_{100}) of a NH_4Cl 1000 p.p.m. standard solution were added to incubation mixtures (final volume 3.1 ml) not containing urease. Corresponding blank mixtures not containing NH_4Cl standard were also run initially. Readings (measured potential – mV) were done after NaOH addition. Thus, the ammonia concentration of test or control samples (in p.p.m.) were calculated by the equation :

$$NH_3 \text{ p.p.m.} = \text{anti log } [(\Delta - T)/\Delta]$$

Where: $\Delta = P_{10}(mV) - P_{100}$ (mV) and T = mV of test or control samples

Experiments were repeated ten times and statistical analysis was performed using the Student t test. Significance was accepted at the $p<0.05$ level.

Results

An urease inhibition curve with different concentrations of phytic acid (Fig. 1) was constructed. The concentrations of 10 and 20 mM of phytic acid were chosen to evaluate the urease protection against copper because higher concentrations significantly inhibit enzyme activity *per se* and other authors have also used concentrations of phytic acid between 1 and 10 mM (Champagne, 1987) or more (Gersonde and Weiner, 1982). Table 1 shows the effectiveness of phytic acid in preventing urease inhibition by Cu^{2+}. The inhibitory effect of this metal is significantly reduced in the presence of phytic acid, a finding which is in agreement with previous findings using this enzyme (urease) (Krawczyk *et al.*, 2000; Jabri *et al.*, 1995).

Discussion

Phytic acid contains six phosphates, it can thus act as a metal chelator to inhibit the generation of highly reactive oxygen species such as hydroxyl free radical (OH) and Cu(I)-hydroperoso complex from H_2O_2. It has been speculated that phytic acid binds tightly to Cu^{2+},

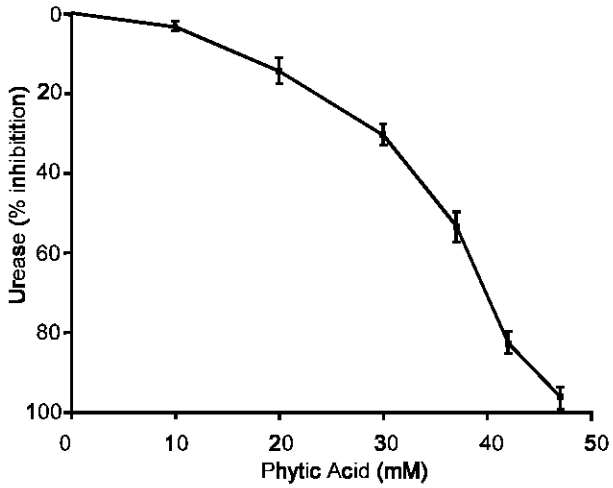

Fig. 1: Inhibition of urease by different concentrations of phytic acid in glycerol-phosphate buffer. Methodological details are described in the text. Results are expressed in % inhibition of the urease activity ± SD (n=10).

preventing the generation of reactive oxygen species (Midorikawa *et al*., 2001). As phytic acid also chelates divalent cations [e.g. calcium (Grases *et al*., 2001), iron (Rimbach and Pallauf, 1998) and nickel (Jabri *et al*., 1995] and urease is the only known nickel-dependent hydrolase enzyme (Jabri *et al*., 1995), perhaps its inhibitory properties against urease are due to ability to chelate nickel (Fig. 1). This explanation is in agreement with Zaborska *et al*., 2001 and Juszkiewicz *et al*., 2004. The chelating properties of the phytic acid are also demonstrated through an ion-selective potentiometry (Champagne, 1987). Our results have also confirmed that phytic acid chelates copper as previously reported (Midorikawa *et al*., 2001; Champagne, 1987). In conclusion, the method described in this report is obviously a feasible one for the determination of phytic acid protection against metals. Moreover, the Cu^{2+} chelating capacity of phytic acid may be useful in preventing interaction of this metal with biomolecules and, accordingly, in lowering the generation of ROS.

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