Phytic Acid

A NATURAL ANTIOXIDANT*

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The catalysis by iron of radical formation and subsequent oxidative damage has been well documented. Although many iron-chelating agents potentiate reactive oxygen formation and lipid peroxidation, phytic acid (abundant in edible legumes, cereals, and seeds) forms an iron chelate which greatly accelerates Fe^{2+}-mediated oxygen reduction yet blocks iron-driven hydroxyl radical generation and suppresses lipid peroxidation. Furthermore, high concentrations of phytic acid prevent browning and putrefaction of various fruits and vegetables by inhibiting polyphenol oxidase. These observations indicate an important antioxidant function for phytate in seeds during dormancy and suggest that phytate may be a substitute for presently employed preservatives, many of which pose potential health hazards.

Phytic acid (myo-inositol hexaphosphoric acid) (Fig. 1) is an abundant plant constituent, comprising 1-5% by weight of edible legumes, cereals, oil seeds, pollens, and nuts (1). The function of these large amounts of phytate has been unclear. The process of synthesizing phytic acid is metabolically expensive, involving six separate ATP-requiring phosphorylations per mol of product. Others have suggested that this substance may serve as a store of phosphorus (2), of cations (3), of glucuronate (a cell wall precursor) (4), or of high energy phosphoryl groups (5), which can be metabolized by phytase and phytate-nucleotide diphosphate phosphotransferases during germination to support early events in plant development. However, many other metabolites may also supply energy, glucuronate, cations, and phosphorus, suggesting that phytate may subserve additional presently unknown functions.

In this regard, we recently observed that phytate, by virtue of chelating free iron, is a potent inhibitor of iron-driven hydroxyl radical (•OH) formation (6). Hydroxyl radical generation mediated by iron requires the availability of at least one coordination site that is open or occupied by a readily dissociable ligand such as water. Although most iron chelates retain a reactive coordination site, Fe^{2+}-phytate does not and consequently fails to support •OH generation (6).

The coordination chemistry of iron phytate chelates with iron-to-phytate ratios exceeding one is unknown due to the low solubility of these polyferric phytate chelates. Both UV-visible spectroscopy and ¹H NMR relaxation measurements used for establishing the relationship between coordination water and the reactivity of iron chelates (6) require large concentrations of iron. Based on the stereochemistry of polyferric phytate complexes, however, they are likely to retain an aquo coordination site and, therefore, to support the Fenon reaction, i.e. the reduction of H₂O₂ by Fe^{2+} to form hydroxyl radicals (7). Nevertheless, Fe(III)-phytate completely blocks •OH formation and strongly suppresses lipid peroxidation. In view of these findings, we thoroughly investigated the effects of phytic acid on the iron redox chemistry. A detailed description of the antioxidant properties of phytic acid is presented in this paper.

MATERIALS AND METHODS

Materials were purchased from the following sources: acetylacetone, adenosine diphosphate, arachidonic acid, bovine serum albumin, dimethyl sulfoxide, dihydroxyphenylalanine, EDTA, hypoxanthine, mushroom tyrosinase, sodium acetate, sodium phosphate, thiorbarbituric acid, trichloroacetic acid, Tris base, and xanthine oxidase from Sigma; FeSO₄ and FeCl₃·6H₂O from J. T. Baker Chemical Co. All other chemicals used were of analytical grade.

In all experiments involving ferrous or ferric iron, the iron solutions were prepared in concentrations >20 times the desired final concentration within 5 min of the time of addition. This was done to prevent spontaneous oxidation of ferrous iron and the formation of insoluble ferric iron hydroxides. No detectable precipitation occurred over the course of the experiments.

The generation of hydroxyl radical (•OH) was determined as described previously (6). Triplicate 1.0-ml samples of 50 mM Tris, pH 7.4, 50 μM Fe²⁺, 500 μM chelating agent, 50 mM dimethyl sulfoxide, 300 μM hypoxanthine, and 18 milliliters of xanthine oxidase (to generate superoxide) were incubated at 37 °C for 30 min. Formaldehyde produced by reaction of •OH with dimethyl sulfoxide (8, 9) was determined spectrophotometrically by the Hansch reaction (10).

Lipid peroxidation was estimated by reacting thiorbarbituric acid with aldehydes such as malondialdehyde which form from the breakdown of polyunsaturated fatty acids (11). Triplicate 1.0-ml samples of 169 μM arachidonic acid, 150 μM Fe²⁺, 1.0 mM sodium acetate, 240 μM chelating agent, and 100 mM NaHCO₃, pH 7.4, were incubated at 30 °C for 90 min. Thiorbarbituric acid-reactive substances ("malondialdehyde") were quantitated spectrophotometrically (11) after precipitation of arachidonic acid by the sequential addition of bovine serum albumin and trichloroacetic acid.

Oxygen utilization was measured in a thermostatted sealed 2.0-ml vessel using a Clark electrode and a Yellow Springs Instrument Co. model 53 oxides monitor. Oxygen tension was monitored continuously on a strip-chart recorder. Reaction conditions are listed in the figure legends. The absolute oxygen content of 50 mM Tris, pH 7.4, at 25 °C was determined titrimetrically using the azide modification of the Winkler method (12).

Tyrosinase activity was assayed by the conversion of dihydroxyphenylalanine to dopachrome as previously described (13). The oxidation of 1.5 mM dihydroxyphenylalanine at 30 °C in 100 mM sodium

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(Received for publication, February 13, 1987)
Similarly, phytate prevents the peroxidation of arachidonic acid driven by ascorbic acid and iron. Substantial amounts of malondialdehyde arise from arachidonic acid in the presence of free iron or of an iron-ADP chelate (Fig. 2B). However, the addition of phytate prevents this iron-dependent generation of malondialdehyde. The magnitude of the effect of chelating agents on 'OH formation does not directly correspond to that on lipid peroxidation. This serves to emphasize that different reactions may be involved in the two processes and that, during lipid peroxidation, iron may catalyze several steps, e.g. 'OH-dependent hydrogen abstraction, 'OH-independent formation of lipid peroxides, and catalysis of the formation of the final aldehydic cleavage products.

Since phytic acid occupies all six coordination sites in Fe(III)-phytate, the chelate cannot participate in the Fenton reaction (6), i.e. phytic acid inhibits 'OH generation and subsequent lipid peroxidation. At higher iron-to-phytate ratios, however, iron is likely to gain an aquo coordination site to support the production of oxyradicals. Perhaps the major antioxidant effect of phytate is in greatly speeding the oxidation of iron by O2, but not by H2O2. As shown in Fig. 3, phytate substantially increases the rate of O2 utilization by Fe2+ in a concentration-dependent manner without affecting the rate of O2 consumption mediated by Fe2+/ascorbate. Fig. 3 demonstrates that phytic acid accelerates the oxidation of Fe3+, whereas it has no effect on the reduction of Fe2+, the rate-limiting step in the removal of O2 by iron and ascorbic acid. This phytate-induced decrease in the iron reduction potential ensures that the steady-state concentrations of Fe2+ are very low.

Fig. 4 further illustrates the large effect of 2 mM phytic acid on the oxidation of 500 µM Fe3+ in 50 mM Tris, pH 7.4, at 25 °C, containing 4.7 ppm oxygen (147 µM) as determined by the Winkler method. Oxidation was complete after only 3 min in the presence of phytate and had consumed 121 µM oxygen, which corresponds to a ratio of 4.13 Fe3+/O2. The net reaction is very likely 4Fe3+ + O2 + 4H+ → 4Fe2+ + 2H2O and probably involves the intermediate formation of O2 and H2O2. The rate of reaction for iron alone agrees fairly well with the rate of Fe2+ oxidation measured spectrophotometrically (6).

The dependence of oxygen utilization on Fe2+ concentration is shown in Fig. 5. The initial rates of oxygen consumption at 1 mM Fe2+ calculated from linear regression analyses are 23.4 and 2026 mm Hg/min in the absence and presence of 2 mM

**RESULTS**

As an initial test of the possibility that phytate might exert an antioxidant effect, we measured the effects of added phytate upon iron-mediated 'OH production and arachidonic acid peroxidation. As shown in Fig. 2A, substantial amounts of 'OH are produced by a superoxide-generating system in the presence of iron alone. Even greater amounts of 'OH are evolved if adenosine diphosphate is added to chelate the iron. Generation of this oxyradical, however, is completely blocked by the addition of micromolar amounts of phytic acid (6); phytate did not affect xanthine oxidase activity as determined by the measurement of the rate of nitroblue tetrazolium reduction by the O2 generated during conversion of purine to oxypurine (14). It is important to note that the inhibition of 'OH generation is found over a wide range of phytate:iron ratios from 1:4 to 29:1 (6). The effect is due to occupation of all iron coordination sites by phytate and does not arise from precipitation of an insoluble iron-phytate complex; all iron-phytate chelates prepared were completely soluble.

**FIG. 2.** Effect of ADP and phytate on iron-mediated 'OH formation (A) and lipid peroxidation (B). The mean and S.D. of three determinations are shown. Generation of hydroxyl radical ('OH) during 30 min in 50 µM Fe3+, 500 µM chelating agent, 50 mM Tris, pH 7.4, 50 mM dimethyl sulfoxide, 300 µM hypoxanthine, and 18 milliunits of xanthine oxidase at 37 °C was determined as previously described (6). Lipid peroxidation was measured by incubating 160 µM arachidonic acid, 150 µM Fe3+, 1.0 mM ascorbate, 240 µM chelating agent, and 100 mM NaHCO3, pH 7.4, at 30 °C for 60 min and determining malondialdehyde (MDA) with thiobarbituric acid spectrophotometrically (11).

**FIG. 3.** Phytate dependence of iron-catalyzed oxygen consumption. All initial rates were determined in 50 mM Tris, pH 7.4, containing 50 µM Fe3+ (A) or 50 µM Fe2+ and 1 mM sodium ascorbate (B) at 25 °C.
Phytic acid: a Natural Antioxidant

Iron-mediated oxidative damage in biological systems has been well documented. Even chelated iron is catalytically active as long as iron within the complex retains an available coordination site (6). As shown above, iron-catalyzed OH formation is enhanced by ADP, while ATP greatly stimulates lipid peroxidation (results not shown). Catalysis of oxidative processes by these two nucleotides, present in high concentration in all animal and plant cells, may account for the burst of oxygen consumption and increased susceptibility to oxidative damage upon tissue injury due to the release of nucleotides and, perhaps, superoxide anion radicals (O2•−). The latter may form OH by the classical Haber-Weiss process (16). There exists ample evidence that much oxidative damage is caused by OH, a highly reactive yet short-lived radical. It is likely to oxidize indiscriminately those molecules nearest to its site of generation.

By virtue of forming a water-excluding monoferryl phytate chelate, phytic acid effectively blocks hydroxyl radical formation and greatly diminishes lipid peroxidation (6). Due to the low solubility of polyferryl phytate chelates, their iron coordination chemistry and existence of dispelacable water have not been investigated. The stereochemistry of polyferryl phytates, however, suggests the availability of a free coordination site, yet these chelates still fail to catalyze the above oxidative events. This antioxidant property arises from the phytate-mediated acceleration of the oxidation of Fe3+ by molecular oxygen, but not by H2O2, while the reduction of Fe3+ to Fe2+ remains unaffected. Thus, phytic acid causes a substantial shift in the redox potential of iron, ensuring the rapid removal of Fe3+ without the concomitant production of phytic acid, respectively. Thus, phytate clearly accelerates Fe2+ → Fe3+, and this perturbation of the equilibrium may help explain the blockade of OH production and lipid peroxidation by phytate.

These results suggested that phytate might actually slow the oxidation of whole plant tissues. Raw potato cubes submerged in tap water turn brown when stored at room temperature overnight, a process which is partially dependent upon oxidative iron-mediated reactions. The addition of 50 mM phytate, pH 7.4, retarded this discoloration for several days; furthermore, it maintained textural firmness and prevented the development of putrid odors, possibly by depriving spoilage microorganisms of iron, a nutritive required for bacterial growth (15). Similar results have been obtained with apples, avocados, and bananas.

This dramatic preservative and antioxidant effect on fruits and vegetables is surprising considering the existence of tyrosinases (polyphenol oxidases) in these materials. Their activation has been shown to be primarily responsible for the browning of many fruits and vegetables upon cutting. Indeed, we find that the phytate concentration used above for the prevention of browning causes a 48% inhibition of mushroom tyrosinase activity (Fig. 6). No attempt was made to understand the mechanism of this inhibition. It may arise from electrostatic interactions between phytate and amino acid residues, as opposed to chelation and removal of the Cu2+ cofactor, since 50 mM EDTA, pH 7.4, had no effect on the enzymatic activity at all. Thus, phytate may retard browning through both diminution of OH production and inhibition of tyrosinase activity. However, because the latter is only partially blocked, more complete preservation of cooked or tyrosinase-free foods by phytate (at greatly reduced concentrations) might be expected where rancidity and deterioration result mainly from iron-catalyzed oxidative damage.

**DISCUSSION**

Iron-mediated oxidative damage in biological systems has been well documented. Even chelated iron is catalytically active as long as iron within the complex retains an available coordination site (6). As shown above, iron-catalyzed OH formation is enhanced by ADP, while ATP greatly stimulates lipid peroxidation (results not shown). Catalysis of oxidative processes by these two nucleotides, present in high concentration in all animal and plant cells, may account for the burst of oxygen consumption and increased susceptibility to oxidative damage upon tissue injury due to the release of nucleotides and, perhaps, superoxide anion radicals (O2•−). The latter may form OH by the classical Haber-Weiss process (16). There exists ample evidence that much oxidative damage is caused by OH, a highly reactive yet short-lived radical. It is likely to oxidize indiscriminately those molecules nearest to its site of generation.

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'OH as in the case of the Fenton reaction. Presumably, a similar diminution in the reduction potential of monoferric phytate occurs, which imparts a dual antioxidant function to phytic acid at high phytate-to-iron ratios, i.e., the inhibition of the Fenton reaction and the fast depletion of Fe^{2+}.

Fe^{2+} alone has been shown to cause the production of oxyradicals and lipid peroxidation, whereas Fe^{3+} is relatively inert, even in the presence of oxygen and polyunsaturated lipids. Numerous biological processes lead to the formation of Fe^{2+} and subsequent 'OH formation. The suppression of these oxidative events ordinarily catalyzed by free iron and many iron chelates may well be an important antioxidant function of the large amounts of phytate within plant seeds, cereals, legumes, nuts, and pollens. Indeed, most of the iron in seeds is complexed by phytate (17), which may help explain the notorious resistance of unsaturated fatty acids of soybean seeds to oxidative stress (18) and the unusually long viability (>1000 years) of some seeds despite the presence of substantial amounts of potentially dangerous iron and unsaturated fatty acids in close proximity.

Of 22 iron chelating agents previously studied, only four displace iron-coordinated water and, thereby, block 'OH generation (6). These are phytate, diethylenetriaminepentaacetic acid, ethylenediamine-di(o-hydroxyphenylacetic acid), and desferrioxamine B methanesulfonate (Desferal). The latter three are either too costly, potentially toxic, or ineffective as preservatives. Furthermore, only phytic acid accelerates O_2-mediated Fe^{3+} depletion while inhibiting the Fenton reaction. Thus, of all readily available iron chelators, only phytate appears to have promise as an effective and nontoxic food preservative. In fact, phytic acid is already being manufactured in large-scale quantities for use in various food applications (19). We believe that phytic acid represents a rational and economical approach to the preservation of a variety of oxygen-sensitive biological materials (20).

REFERENCES