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The effect of fruit extracts with polyphenol oxidase (PPO) activity on the in vitro accessibility of iron in high-tannin sorghum

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Abstract

Dephytinized high-tannin sorghum flour was incubated with crude extracts from pear, banana or avocado, respectively, followed by investigation of the effects on the phenolic content and on in vitro accessible iron. All fruits contained polyphenol oxidase (PPO) activity and incubation resulted in significant reduction of phenolic compounds. Incubation with avocado extract resulted in the lowest levels of phenolic compounds, as well as the highest amount of in vitro accessible iron. Peroxidase activity and some organic acids in the fruit extracts might also have contributed to the positive effect on iron accessibility. Nevertheless, incubation of the sorghum flour with the fruit extracts under conditions enabling the PPO to oxidize phenolic compounds, resulted in the highest accessibility of iron. The results from this study suggest that the PPO activity in simple fruit extracts can be utilized to increase the accessibility of iron in dephytinized polyphenol-containing cereal foods.

Keywords: Oxidation; Polyphenol oxidase; PPO; Fruits; Sorghum; Tannin; Polyphenol; Iron accessibility; Bioavailability; Peroxidase; Organic acids

1. Introduction

Polyphenol oxidase (PPO) is often associated with deterioration of foods because of its involvement in browning reactions. It oxidizes a number of phenolic compounds to the corresponding quinones, which easily undergo secondary reactions with amino acids, proteins or other phenols, to form melanin pigments (Friedman, 1996; Whitaker & Lee, 1995). The ability of PPO to oxidize phenolic compounds may, however, be utilized to increase the bioavailability of iron in polyphenol-containing plant foods. Coloured cereals, for example, are known to contain large amounts of phenolic compounds, such as condensed tannins. High-tannin sorghum is used as a staple food in many arid areas of the world and the tannins contribute strongly to the low bioavailability of iron in the vegetable diet eaten by the people in these areas. It is mainly the *ortho*-dihydroxyl groups present in condensed tannins that have been shown to bind iron (III) (Gust & Suwalski, 1994; Slabbert, 1992), making it unavailable for absorption in the gastrointestinal tract (Brune, Rossander, & Hallberg, 1989). Oxidation of phenolic compounds may result in a reduced iron-binding capacity and a higher availability of iron. We have shown, in an earlier study, that the in vitro accessibility of iron in phytate-reduced high-tannin varieties of sorghum and millet increased with polyphenol oxidase (mushroom tyrosinase) treatment (Matuschek, Towo, & Svanberg, 2001).

PPO may not be able to oxidize complex phenolics such as oligomers and polymers, except in the presence of a simpler phenolic compound (Cheynier & da Silva, 1991; Haslam et al., 1992). A hydroxycinnamic acid, for example, can trigger the oxidation of the more complex polyphenol by a coupled redox reaction. This has been shown, e.g., in procyanidins (Cheynier & da Silva, 1991) and theaflavins (Opie, Clifford, & Robertson, 1993). In addition to condensed tannins, high-tannin sorghum also contains flavonoids and phenolic acids (Hahn, Rooney, & Earp, 1984), which probably can be used as initial substrates by PPO and thereby contribute to oxidation of the tannins.

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Many fruits and vegetables, such as pear, banana and avocado, have a high PPO activity (Almeida & Nogueira, 1995). In addition, fruit extracts also contain significant amounts of organic acids, which may have a positive effect on iron absorption (Ballot et al., 1987; Gillooly et al., 1983). However, fruit juices are often ingested at the same time as a meal, which will not enable the PPO to reduce the phenolic content in the food. The purpose of this study was to investigate how incubation of dephytinized high-tannin sorghum with fruit extracts containing PPO activity affects the phenolic content and the in vitro accessible iron.

2. Materials and methods

2.1. Materials

A high-tannin sorghum (*Sorghum bicolor* L. Moench) variety, locally called udo, was purchased at a local market in Dodoma, Tanzania. The grains were sorted and washed several times with tap water, followed by repeated rinsing in deionized water. After washing, the grains were dried and milled in a disc-type mill (Laboratory Mill 3300) to obtain a fine flour (80% passing a 250 μm sieve). Ripe fruits of pear (*Pyrus communis*), banana (*Musa* sp.) and avocado (*Persea americana*) were purchased at a local store in Göteborg, Sweden. Wheat phytase (EC 3.1.3.26, P1259), pepsin (EC 3.4.23.1, P6887), pancreatin (P1750), bile extract (B8631), (±)-catechin (C1788), 4-methylcatechol (M34200) and 2,3-dihydroxynaphthalene (D116009) were purchased from Sigma–Aldrich, Stockholm, Sweden.

2.2. Phytase incubation

A portion of sorghum flour was suspended in H_2O (10 ml/g flour) and the pH was adjusted to 4.8 with HCl before addition of lyophilized phytase (2.0 U/g flour). The mixture was incubated in a shaking water bath at 55 °C for 24 h, followed by freeze-drying.

2.3. Crude fruit extracts

PPO was extracted by mixing 100 g fruit pulp in pieces with 100 ml ice cold H_2O (150 ml H_2O/100 g for avocado) and 1 g polyvinylpyrrolidone in a Braun 4262 food processor (Braun, Kronberg, Germany) for 2 min. The extract was centrifuged at 12,000*g* and 4 °C for 15 min and filtered trough a double cotton cloth. The filtrate was dialyzed (Spectra/Por 4, MWCO 12–14 kDa, Spectrum Laboratories, Rancho Dominguez, US) against H_2O at 4 °C for 48 h and kept at −20 °C until used.

2.4. Determination of PPO activity

The PPO activity in the fruit extracts was determined by both polarographic and spectrophotometric methods. The polarographic determinations were made on a Hansatech Oxygraph system equipped with a Clark-type oxygen electrode fitted in a pre-heated cell (30 °C) with a gas-tight plunger (Techtum Lab, Umeå, Sweden). The fruit extract (50 μl) was added to 2 ml of 20 mM 4-methylcatechol in 0.1 M phosphate buffer at pH 6.0. Prior to the measurement, 4-methylcatechol was dissolved in a small amount of methanol and added to the buffer solution, which had been bubbled with air at 30 °C for 1 h. The PPO activity was expressed as nmol O_2 consumed per min and per ml of enzyme solution. The spectrophotometric determinations were made at 500 nm and the activity was calculated from the initial change in absorbance. The substrate (300 μl of 0.2 M 4-methylcatechol in methanol) was mixed with 2.6 ml 0.1 M phosphate buffer at pH 6.0 and 100 μl fruit extract were added immediately before the measurement. One unit of PPO activity was defined as equal to a change of 0.001 in absorbance per min and per ml of enzyme solution.

2.5. Detection of peroxidase activity

Hydrogen peroxide (0.5%, 100 μl) and guaiacol (1% in 95% ethanol, 100 μl) were added to 500 μl of the fruit extracts. Peroxidase activity was estimated visually after 3.5 min by the formation of a red–brown reaction product. A strong colour reaction was interpreted as a high peroxidase activity.

2.6. Incubation with fruit extracts

A sample of dephytinized sorghum flour was suspended in fruit extract (10 ml/g flour). The pH was adjusted to 6.5 with 1 M NaOH and the slurry was incubated in a shaking water bath at 30 °C for 16 h. The sample was freeze-dried before analysis of phenolic compounds. A control sample for in vitro accessible iron was made by incubation of dephytinized sorghum with fruit extract containing 30 mM 2,3-dihydroxynaphthalene (2,3-DHN), which was dissolved in a small amount of methanol before addition to the extract.

2.7. Determination of organic acids

The amount of ascorbic acid in the fruit extracts was determined on an HPLC system equipped with a Kromasil C18 column (5 μm, 150 mm × 2.1 mm, Akzo Nobel, Bohus, Sweden). The mobile phase consisted of A: 4 mM tetrabutyl ammonium hydroxide with a pH value adjusted to 4.1 with formic acid and B: 25% ace-

tonitrile, at a ratio of A:B 2:3. The flow rate was 0.8 ml/min, the injection volume was 20 μl and ascorbic acid was detected by a UV detector at 255 nm. Before analysis, the samples were diluted 1:10 with H_2O and all samples and standards contained 0.01% dithiothreitol (DTT). The amounts of citric, tartaric, malic, succinic, lactic, acetic and propionic acid in the fruit extracts were determined by ion exclusion chromatography on an HPLC system equipped with an Amniex HPX-87H column (BioRad Laboratories) at 65 °C. The acids were detected by a UV detector at 210 nm. Sulfuric acid (0.008 M) was used as mobile phase. The flow rate were 0.6 ml/min and the injection volume was 50 μl. Before analysis, 500 μl of the fruit extracts was put on a 100 mg Isolute SAX column (International Sorbent Technology, Mid-Glamorgan, UK), eluted with 750 μl 0.5 M HCl and diluted to 5 ml with H_2O.

2.8. Determination of total iron content

The total amount of iron in the sorghum flour was determined by wet acid digestion in teflon containers at a temperature of 180 °C using an Ethos Plus microwave system (Milestone, Bergamo, Italy). To 0.3 g of cereal flour were added 3 ml H_2O, 0.15 ml 37% HCl and 0.75 ml 65% HNO_3, which was followed by digestion with a temperature programme reaching 180 °C in 15 min and holding that temperature for 20 min. After cooling to room temperature, the samples were transferred to test tubes and diluted to 10 ml with H_2O. The following day, 100 μl ascorbic acid solution (20 g/l) were mixed with 900 μl sample and this solution was analyzed for total iron by HPIC, as described by Fredrikson, Carlsson, Almgren, and Sandberg (2002).

2.9. Determination of in vitro accessible iron

In vitro iron accessibility was measured as iron solubility under physiological conditions, according to the method of Svanberg, Lorri, and Sandberg (1993) with some minor modifications. A 0.5-g sample of flour was suspended in 5 ml H_2O, followed by addition of 5 ml of a pepsin solution (0.3% pepsin in 0.1 M HCl). In addition to pepsin, the solution also contained physiological amounts of Na (49 mM as NaCl), K (12 mM as KCl), Ca (10 mM as $CaCl_2 \cdot 2H_2O$), Mg (2.4 mM as $MgCl_2 \cdot 6H_2O$) and phosphate (3.5 mM as KH_2PO_4) (Diem & Lentner, 1975). The pH was adjusted to 2.0 with NaOH and the mixture was incubated in a shaking water bath at 37 °C for 90 min. After incubation, 1.5 ml of a pancreatin and bile solution (6 mg pancreatin and 37.5 mg bile extract in 0.1 M $NaHCO_3$) were added. The pH was adjusted to 5.0 with NaOH, and the mixture was incubated for an additional 30 min. After adjustment of pH to 6.0 with NaOH the mixture was centrifuged at 4300*g* for 20 min and the supernatant was filtered through a 45-μm filter. The pH in the filtrate was lowered by addition of 100 μl 0.5 M HCl to 800 μl sample, followed by addition of 100 μl ascorbic acid solution (20 g/l). After 10 min, the samples were centrifuged (11,000*g*, 10 min) and the clear solution obtained was analyzed for soluble iron, including free soluble complexes of iron, by HPIC as described by Fredrikson et al. (2002). The amount of soluble iron in the supernatant was expressed as a percentage of the total amount of iron in the sample.

2.10. Determination of phytate and phenolic content

Phytate (inositol-6-phosphate) and lower inositol phosphates were extracted during stirring for 3 h with 0.5 M HCl (0.5 g sorghum flour/10 ml HCl) and determined according to the method of Carlsson, Bergman, Skoglund, Hasselblad, and Sandberg (2001). The phenolic compounds were extracted during stirring for 3 h with methanol containing 1% conc. HCl (50 mg sorghum flour/10 ml acidified methanol) and determined by the Prussian Blue assay as described by Price and Butler (1977).

2.11. Statistical analysis

Differences in mean values were tested by analysis of variance (ANOVA) and significance levels were obtained with Tukey's multiple range test (Wilkinson, 1990). A significance level of <0.05 was used. Simple regression analysis was performed to correlate the phenolic content with the in vitro accessible iron.

3. Results

3.1. The food system

We tried to keep the food system, consisting of sorghum flour and fruit extracts, low in factors that are known to interfere with iron solubility. The sorghum flour did not contain any detectable amounts of phytate or lower inositol phosphates (<0.05 μmol/g) after incubation with phytase. Additionally, dialysis of the fruit extracts resulted in effective reduction of organic acids. Neither ascorbic, citric, tartaric, malic, succinic, lactic, acetic or propionic acids could be detected in the pear extract after dialysis. However, the banana extract contained about 20 ppm citric acid after dialysis and the dialyzed avocado extract contained about 400 ppm succinic acid. The fruit extracts contained low amounts of soluble phenolic compounds, 33, 36 and 87 μg CE/ml for pear, banana and avocado, respectively. The contribution of phenolic compounds from the fruit extracts was negligible compared with the phenolic content in the sorghum flour.

3.2. Enzyme activity in the fruit extracts

The PPO activities in the fruit extracts are shown in Table 1. Banana had the highest PPO activity, as measured by the polarographic and the spectrophotometric methods, respectively, and the activity in the avocado extract was approximately half that in the banana extract. In contrast, the pear extract had a much lower PPO activity. The PPO activities in the banana and the avocado extracts were as much as 49 and 25 times higher, respectively, than the activity in the pear extract when measured as oxygen consumption. Peroxidase activity was detected in all fruit extracts by visual estimation of coloured reaction products. The activity was lowest in pear, closely followed by banana, while the avocado extract had a much more intense colour formation, implying a high peroxidase activity.

3.3. Inhibitory effect of 2,3-dihydroxynaphthalene

The inhibitory effect of 2,3-dihydroxynaphthalene (2,3-DHN) on PPO activity was examined in two ways. First, the fruit extracts showed no PPO activity with the spectrophotometric method when 30 mM of the inhibitor was added to the substrate solution. It was also observed that no coloured reaction products were formed during incubation of the sorghum flour with fruit extracts containing 2,3-DHN, in contrast to incubation with fruit extracts alone. Unfortunately, it was not possible to measure the effect of addition of 2,3-DHN on the amount of phenolic compounds present in the sorghum samples, since the inhibitor also reacted with the reagents used for determination of the phenolic content.

3.4. Effect of incubation with fruit extracts on the phenolic content

Incubation of dephytinized sorghum flour with fruit extracts resulted in significantly lower amounts of phenolic compounds (Table 2). The largest reduction in phenolic compounds (50%) was obtained after incubation with avocado extract, with banana extract being almost as effective (43% reduction). Incubation with pear extract also significantly ($p < 0.05$) reduced the amount of phenolic compounds, but by approximately 25%.

3.5. Effect of incubation with fruit extracts on the in vitro accessible iron

The in vitro accessible iron increased when dephytinized sorghum flour was incubated with fruit extracts (Fig. 1) and the accessible iron showed a high inverse relationship with the phenolic content ($r = -0.956$). The

Table 1
Polyphenol oxidase (PPO) activity in the fruit extracts as measured by the polarographic (A) and the spectrophotometric (B) methods, respectively[a]

Fruit extract	PPO activity (A) O_2 uptake[b]	PPO activity (B) product formation[c]
Pear	0.448	0.292
Banana	22.0	9.60
Avocado	11.2	4.65

[a] Mean values of duplicate samples are shown.
[b] 10^3 nmol O_2 consumed min^{-1} ml^{-1}.
[c] 10^3 U.

Table 2
Effect of incubation with extracts from pear, banana or avocado, respectively, on the phenolic content of dephytinized high-tannin sorghum[a]

	Phenolic content[b]
Dephytinized sorghum	12.0 ± 0.512^{A}
+Pear extract	9.01 ± 0.395^{B}
+Banana extract	6.88 ± 0.397^{C}
+Avocado extract	5.98 ± 0.402^{C}

[a] Mean values ± SD of triplicate samples are shown. Figures followed by different letters are significantly different ($p < 0.05$).
[b] mg catechin equivalents (CE)/g dry weight.

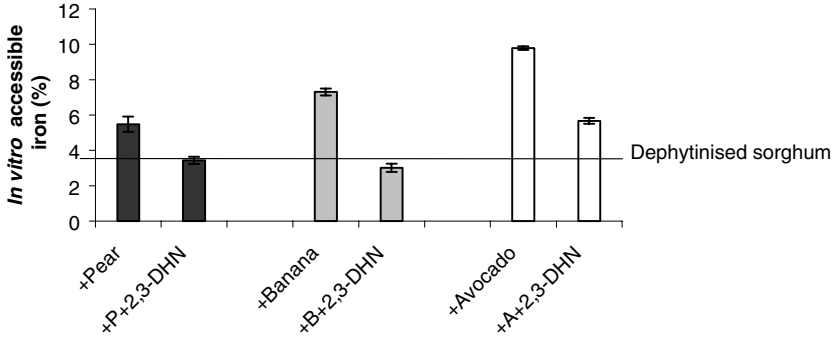

Fig. 1. Effect of incubation with extracts from pear, banana or avocado, respectively, on in vitro accessible iron in dephytinized high-tannin sorghum. Fruit extracts containing 30 mM 2,3-dihydroxynaphthalene (2,3-DHN) were used as control samples. Mean values ± SD of triplicate samples are shown.

most pronounced effect was obtained after incubation with avocado extract, which increased the accessible iron more than 1.7-fold. Incubation with pear or banana extract resulted in approximately 50% and 100% higher accessibility, respectively. Addition of 30 mM 2,3-DHN to the fruit extracts before incubation resulted in lower amounts of accessible iron. The in vitro accessible iron, after incubation with pear and banana extract containing 2,3-DHN, was not significantly different from the dephytinized sorghum ($p > 0.05$). Incubation with avocado extract containing 2,3-DHN did, however, result in an accessibility that was significantly higher than in the dephytinized sorghum.

4. Discussion

The bioavailability of iron is often very low in cereal-based meals ($\leqslant 5\%$) because of the high content of phytate and/or polyphenolic compounds (tannins). Dephytinization alone is not sufficient to increase the bioavailability of iron from cereals that contain high amounts of polyphenolic compounds. This was recently demonstrated in a study by Hurrell, Reddy, Juillerat, and Cook (2003), where almost complete dephytinization of high-tannin sorghum did not increase iron absorption from a porridge meal. In contrast, degradation of phytate in low-tannin sorghum, as well as in other cereals, such as wheat, maize and oats, resulted in an increased iron absorption. We have earlier shown that oxidation of phenolic compounds with a commercial polyphenol oxidase (mushroom tyrosinase) increases the in vitro accessibility of iron in phytate-reduced high-tannin sorghum (Matuschek et al., 2001). In the present study, incubation of dephytinized high-tannin sorghum with crude water extracts from pear, banana or avocado reduced the phenolic content and resulted in an increased in vitro accessible iron. In addition to the enzymatic activity in the fruit extracts, it is probable that the natural content of organic acids also has a positive effect on iron accessibility.

Pear, banana and avocado are fruits that easily turn brown when cut surfaces are exposed to air, which shows that they contain highly active PPO. Among the varieties used in this study, banana had the highest PPO activity (Table 1). Consequently, incubation of high-tannin sorghum with banana extract effectively reduced the phenolic content (Table 2). However, the reduction in phenolic compounds after incubation with avocado extract was of the same order of magnitude, even though the PPO activity in the avocado extract was much lower. It should be noted, though, that in order to facilitate the extraction, the H_2O/fruit ratio in the avocado extract was 1.5 times the ratio in the pear and the banana extract. Nevertheless, there was a large reduction of phenolic compounds after incubation with the avocado extract, which may be explained by an additional effect of peroxidase activity. Peroxidase is able to oxidize phenolic compounds in the presence of hydrogen peroxide (Richard Forget & Gauillard, 1997), which may be formed as a byproduct during the PPO-catalyzed oxidation (Jiang & Miles, 1993). The avocado extract showed a much higher peroxidase activity than the pear and the banana extracts. Peroxidase activity may therefore have contributed to the large reduction of phenolic compounds obtained after incubation with the avocado extract. The results regarding the inhibitor, 2,3-dihydroxynaphthalene (2,3-DHN), further suggest that the peroxidase activity contributed to this reduction. Addition of 2,3-DHN to the avocado extract before incubation resulted in an iron accessibility that was significantly higher than in the sorghum flour (5.67% compared with 3.59%). The inhibitor is specific for PPO (Mayer, Harel, & Shain, 1964; Zawistowski, Biliaderis, & Murray, 1998), and probably had no effect on the peroxidase activity during the incubation.

Incubation with avocado extract resulted in the highest in vitro accessible iron, 9.79% (Fig. 1). In addition to the high peroxidase activity in the avocado extract, the presence of other factors, e.g., organic acids, may have a positive effect on iron solubility. The correlation between the in vitro accessible iron and the phenolic content was higher when the results from incubation with the avocado extract were excluded ($r = -0.996$ vs. -0.956), which implies that some other factors in that extract affected the iron accessibility (Fig. 2). The avocado extract contained 400 ppm succinic acid after dialysis and Salovaara, Sandberg, and Andlid (2002) showed that succinic acid increased ferric iron absorption in a Caco-2 cell system at concentrations higher than 235 ppm (2 mM). Suzuki, Clydesdale, and Pandolf (1992) also showed that succinic acid increased iron solubility in a model system containing lignin, even though the effect was lower than, for ex-

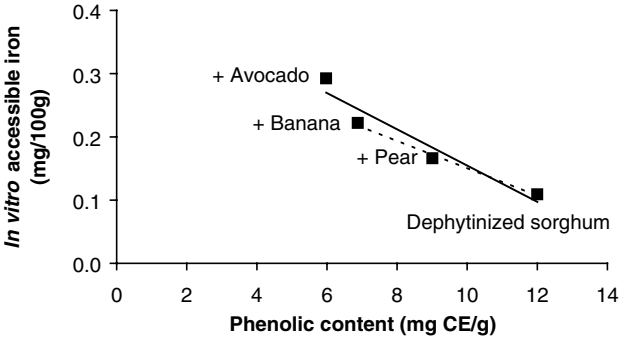

Fig. 2. Relationship between the phenolic content and the in vitro accessible iron in dephytinized high-tannin sorghum after incubation with fruit extracts. (- - -) shows the relationship when the results from incubation with avocado extract are excluded. Mean values of triplicate samples are shown.

ample, ascorbic and citric acid. In the present study, addition of 400 ppm succinic acid to the dephytinized sorghum did not increase the in vitro accessible iron (3.12% accessible iron with succinic acid compared with 3.59% without succinic acid). Likewise, addition of 20 ppm citric acid, similar to what was found in the banana extract after dialysis, did not increase the accessible iron (3.33% accessible iron with citric acid). It is possible, however, that succinic and citric acid affected the in vitro accessible iron when they were present in the fruit extracts. It is important to note, though, that there are studies that show both positive and negative effects of citric acid on iron absorption (Ballot et al., 1987; Gillooly et al., 1983; Hallberg & Rossander, 1984) Citric acid forms strong complexes with iron and may thereby be reluctant to donate iron to the receptors in the mucosal brush border during the absorption process (Narasinga Rao & Subba Rao, 1992).

The effect of organic acids on in vitro accessible iron was further elucidated in this study by incubation of dephytinized high-tannin sorghum with banana extract that had not been dialyzed. Incubation with non-dialyzed banana extract resulted in as much as 12.5% accessible iron, which is about 70% higher than when the dialyzed banana extract was used. Before dialysis, the banana extract contained high amounts of citric and malic acid, as well as significant amounts of ascorbic, propionic and tartaric acids, which probably contributed to the higher in vitro iron accessibility. The enzyme activities were, however, also higher before dialysis, but it is difficult to draw a conclusion about the effect of an even higher PPO activity on the iron accessibility, since product inhibition of the enzyme has been demonstrated (Le Bourvellec, Le Quéré, Sanoner, Drilleau, & Guyot, 2004; Whitaker, 1994).

Even though organic acids and peroxidase activity probably contributed to the high accessibility of iron obtained after incubation with the fruit extracts, it is clear that the PPO activity in the extracts had a significant effect on the accessibility. The enzyme activity measurements showed that 2,3-DHN effectively inhibited PPO and incubation with fruit extracts containing 2,3-DHN resulted in significantly lower levels of accessible iron (Fig. 1). The inhibitor itself shows iron-binding properties, but addition of 2,3-DHN to dephytinized sorghum flour did not decrease the in vitro accessible iron. The inhibitor was, furthermore, added directly to the fruit extracts, enabling the enzyme and the inhibitor to interact before addition to the sorghum flour. The effect of the incubation per se was also examined by measuring the iron accessibility when banana extract was added after addition of the pepsin solution. During the in vitro digestion process, the PPO in the extract will not be active during the first incubation step because of the low pH value (2.0). However, after raising the pH to 5.0, PPO will probably have a high activity. Addition of the banana extract after the pepsin solution resulted in a higher in vitro accessible iron compared with the dephytinized sorghum, but the accessibility was lower than when the flour was incubated with the extract before the in vitro digestion ($p = 0.001$). These results also support the assumption that incubation of high-tannin sorghum with fruit extracts containing PPO activity significantly increases the in vitro accessible iron. The products formed during enzymatic oxidation of phenolic compounds are difficult to measure, especially when the substrates are complex and somewhat undefined, as in sorghum. Regardless of the final structure, modifications of the functional phenolic groups during oxidation will probably affect the iron-binding capacity, and the results from this study imply that the products formed have a lower iron-binding capacity than the original phenolic compounds.

To conclude, crude extracts from different fruits can be used, in combination with dephytinization, to effectively increase the accessibility of iron in high-tannin cereals. When high-tannin cereal flours were incubated with fruit extracts, the polyphenol oxidase in the extracts was found to reduce the phenolic content of the cereals. The content of organic acids in the fruit extract, as well as a high peroxidase activity, may further increase the iron accessibility. Many fruits in low-income countries contain PPO activity, which makes them suitable for the proposed incubation process. In combination with simple methods that degrade phytate, incubation with fruit extracts could be used at a household level to increase the bioavailability of iron in high-tannin staple cereals.

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