

Minerals and Phytate in the Analysis of Dietary Fiber from Cereals. III

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ABSTRACT

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Bread dough made from wheat flour and bran, with a dietary fiber content of 24% dry matter, was fermented up to 17 hr and baked. During the process, changes were measured in content of dietary fiber and phytate and mineral association to these components *in vitro*. Total dietary fiber content was not influenced by fermentation or baking, even though a slight decrease in soluble fiber and increase in insoluble fiber components was observed. Ash was mainly associated with the soluble fiber fraction. During fermentation and breadmaking, there was a decrease in fiber-associated ash. Even with extremely long fermentation it was impossible to obtain complete hydrolysis of phytate; after 17 hr it was reduced only to half of that present in the dough just before fermentation. Nearly three quarters of the phytate

was detectable in the soluble fiber fraction, but there was none in the insoluble fiber fraction. Specific analysis showed that minerals behave differently, both in binding to fiber fractions before fermentation and in behavior during fermentation. Up to 60% of the total iron was associated with components other than phytate in the dietary fiber complex. Only 10% of the total iron seemed to be associated with phytate. Zinc (24%) and calcium (60%) seemed to be associated with the phytate in the soluble fiber fraction and were released during fermentation. Nine percent of the magnesium was also found associated to this fraction and released during fermentation. All phosphorus in the soluble fiber fraction seemed to be present as phytate.

The content of minerals, dietary fiber, and phytate increases with increasing extraction rate of the flour. Phytate is known to have chelating properties and to form complexes with divalent cations. Other factors in the dietary fiber complex have also been pointed out as possible chelators (Reinhold et al 1976a and b, Ismail-Beigi et al 1977a and b). Reinhold et al (1981) have shown that dephytinized wheat bran binds iron as effectively as native bran. Bread made from dough fermented for 15 hr showed increased zinc absorption compared to bread made without fermentation (Nävert et al 1984).

We have shown (Schweizer et al 1984, Frølich et al 1984) that considerable amounts of ash were associated with dietary fiber components, mainly in the soluble fiber fraction, where most of the phytate was recovered also. Only a small percentage of minerals occurred in the insoluble fiber fraction, except that up to 40% of iron was recovered in this fraction, and no phytate could be detected.

Preparation of leavened bread with a yeast fermentation step allows yeast and wheat phytases to hydrolyse phytic acid. Ranhotra et al (1974) found that all of the phytate in wheat bread was hydrolyzed during baking, which is in contrast to the study of

Harland and Harland (1980). In the present study we investigated the association of minerals to the different fiber fractions *in vitro* during fermentation and baking. This could give an indication to which components in the dietary fiber the minerals were associated. We also looked for any changes in dietary fiber components during these processes.

MATERIALS AND METHODS

Samples

Two independent bread doughs were made from the same ingredients and recipe containing whole grain flour and bran: 700 ml water, 700 g whole grain wheat flour, 300 g wheat bran, 17.5 g salt, and 35 g yeast. The dough was fermented 2-17 hr and then baked for ¾ hr.

Samples were taken at intermediary times during breadmaking: at time zero (directly after mixing the ingredients), A₁; after 2 hr of fermentation, A₂; after 8 hr of fermentation, A₃; after 17 hr of fermentation, A₄; and after 17 hr of fermentation with an additional baking at ¾ hr, A₅. Analyses were made in duplicate on the five samples from the two different doughs. Values in the tables are means from the two bread doughs.

Sample Preparation

All samples were immediately dried in a Phillips microwave oven, HN 1104 A, for 20 sec on full power, with an additional hour at 105°C in a traditional oven. The samples were ground in a Cyclotec sample mill (Tecator AB, Höganäs, Sweden) to a particle

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size of less than 0.45 mm and kept in closed containers. All samples were analyzed for phytic acid, insoluble and soluble dietary fiber, ash, and specific mineral content. Ash content was determined by ashing at 500°C to constant weight.

Mineral Determination

After ashing, the samples were dissolved in a 1:1 solution of hydrochloric and citric acid, for a final concentration of 4% v/v of both acids. The individual minerals were then determined by plasma emission spectrophotometry using a direct reading spectrometer, Simultaneous Instrument, no. 975 1 CAP (manufactured by Jarrell-Ash).

Dietary Fiber

The method used to determine dietary fiber content was based on Hellendoorn's enzymatic, gravimetric method (Hellendoorn et al 1975) as described by Asp et al (1981, 1983). To remove starch completely, an extremely heat-stable α -amylase (Termamyl 60L, Novo, Copenhagen, Denmark) was used in an initial gelatinization step at 100°C for 15 min. Further enzyme digestions were performed with pepsin (Merck no. 7190) at pH 1.5 for 1 hr and with pancreatin (Sigma no. P-1750) at pH 6.8 for 1 hr at 40°C. The mineral content of the enzyme preparations used for a 1-g sample (dry matter) of bread dough was as follows: Ca 0.46, Mg 0.06, P 0.20, Fe 0.001, Zn 0.001 mg per 100 μ l Termamyl; Ca 0.05, Mg 0.14, P 0.58, Fe 0.003, Zn 0.002 mg per 100 mg pepsin; Ca 1.50, Mg 0.06, P 1.00, Fe 0.007, Zn 0.014 mg per 100 mg pancreatin.

Two buffer systems were used in the studies of mineral association, phosphate buffer and citrate buffer. The final buffer concentration before ethanol precipitation was 25 mM for both systems to obtain coprecipitation of phosphorus and other minerals as low as possible (Schweizer et al 1984, Frølich et al 1984). For the same reason the Termamyl step was omitted in these studies.

The fiber components were recovered either by filtration, for determination of dietary fiber content, or centrifugation, in studies of mineral association. For fiber content studies, insoluble fiber components were recovered by filtration and soluble components by precipitation with four volumes of 95% ethanol (final concentration 78%, v/v) followed by filtration, using phosphate buffer in the assay. The filtration was carried out with Tecator's Fibertec system (Tecator AB, Höganäs, Sweden) using 0.5 g of Celite 545 as a filter aid.

In studies of mineral association, insoluble fiber components were recovered by centrifugation for 30 min at 1,600 \times g in a Beckman centrifuge J2-21 at 4°C. The supernatants and washings (100 ml) were precipitated with four volumes of ethanol, and the soluble fiber components were recovered by centrifugation in the same centrifuge for 30 min at 3,000 \times g at 4°C. Citrate buffer was used in the fiber assay with centrifugation to avoid coprecipitation of phosphate.

The dietary fiber values reported in the tables are means of duplicate gravimetric analyses. Values are given in percent of dry matter, if nothing else is indicated, corrected for protein (Kjeldahl N \times 6.25), and ash associated with the fiber.

Dialysis

In some experiments, the soluble fiber was recovered by dialyzing the supernatant obtained after centrifuging the enzyme digest. The

solutions were dialyzed against 4 L double-distilled water for 48 hr at 4°C in Spectrapor sacks (Spectrum Medical Industries, Inc., Los Angeles, CA) with an exclusion limit of 6,000–8,000 daltons. The sack's contents were lyophilized. In this experiment Termamyl was excluded from the assay, because of the rather high mineral content of this enzyme preparation. This procedure was used to avoid the possible structural alteration of soluble fiber or phytate obtained by precipitation with alcohol followed by redissolution before dialysis, which has been pointed out in a previous study (Frølich et al 1984).

Phytate

Insoluble and soluble fiber components of the five samples recovered by centrifugation were analyzed for phytate. Phytate was determined with Holt's method (Holt 1955), which is based on complex formation of phytic acid and Fe (III) ions at pH 1–2. Excess of Fe (III) present in the solution will react with thiocyanate ions to form a characteristic pink complex, Fe(SCN)₃. The optical density at 465 nm in an amyl-alcohol layer is measured, and an inverse linear relation is found for phytate concentrations ranging from 40–200 nmol/L.

Phytase Preparation

The enzyme phytase was prepared from wheat according to the method of Peers (Peers 1953) to obtain an enzyme with optimal activity. The enzyme was kept in a freezer (–20°C).

Phytase Incubations

Some of the supernatants obtained after the first centrifugation of the enzyme digest containing the soluble fiber components were incubated for 18 hr with phytase at 37°C. The enzyme reaction was stopped with 0.1M trichloroacetic acid (TCA) and adjusted back to pH 4.5 with NaOH. The incubate was analyzed for phytate and dialyzed. The contents of the dialyzing sacks were analyzed for phytate and specific minerals after the dialysis.

Statistical Evaluation

The precision of the analysis of the different methods was calculated according to the following equation:

$$s^2 = \frac{1}{2}k \sum_{i=1}^k (x_{i1} - x_{i2})^2$$

where s = standard deviation, k = number of samples, two analyses per sample, x_{i1} and x_{i2} = duplicate values for sample i .

RESULTS AND DISCUSSION

Dietary Fiber Content in Dough and Bread

Table I gives dietary fiber content of the five different bread doughs, which was around 24% dry matter. In Norway the bakers are trying to produce bread with different contents of whole grain flour to increase the amount of dietary fiber, minerals, and vitamins. Most frequently dietary fiber content of bread is around 8–9%, but some bakers are also trying to produce bread with a much higher percentage. By adding bran to the bread dough, it is possible to increase the dietary fiber content considerably, as has been shown in this paper. Total dietary fiber content was not influenced by fermentation or baking, but there seemed to be a slight loss of soluble fiber components. A slight tendency to

TABLE I
Content of Dietary Fiber in Bread Dough After Different Fermentation Periods^a

Samples	Fiber Fraction			Residual Protein		Residual Ash	
	Insoluble	Soluble	Total	Insoluble	Soluble	Insoluble	Soluble
A ₁	21.3	2.4	23.7	3.8	1.5	≤0.2	3.1
A ₂	21.8	2.5	24.3	4.7	1.8	≤0.2	2.5
A ₃	21.7	2.4	24.1	4.1	2.0	≤0.2	2.5
A ₄	22.2	2.1	24.3	2.9	1.5	≤0.2	1.8
A ₅	22.4	1.5	23.9	3.3	1.5	≤0.2	3.3

^a All figures are in percent of dry matter in the dough. The fiber figures are corrected for residual protein and ash. The figures are a mean of two independent experiments, the samples are analyzed in duplicate. The standard deviation of the method is 0.32 g/100 g (Asp et al 1983).

increase insoluble fiber components was also seen, which could be explained by formation of resistant starch (Johansson et al 1984). It has also been pointed out (Theander 1983) that the content of lignin-like polymers might be increased with increasing temperature.

Protein and Ash Associated with Dietary Fiber

The main part of protein (determined as Kjeldahl N \times 6.25) associated with the dietary fiber occurred in the insoluble fraction. During fermentation and breadmaking, the amount of associated protein seemed to be constant both for insoluble and soluble fiber components. In contrast, the ash associated with dietary fiber occurred mainly in the soluble fiber fraction, as pointed out in previous papers (Frølich and Asp 1980, Frølich et al 1984). Ash decreased with increasing fermentation time, which also was true for individual minerals (Table II). Baking seems to reassociate some of the minerals. This is shown both for total ash and individual minerals.

Phytate

Table III shows the fate of phytate during fermentation and baking, both in complete bread dough and in different fiber fractions. After 17 hr of fermentation, the phytate was reduced to about half of that present in the dough at time zero. Even with the longest fermentation it was impossible to obtain complete hydrolysis of phytate, which is in accordance with the findings of Harland and Harland (1980).

During baking an additional breakdown of phytate occurred. The final amount was about 40% of the original.

Before fermentation nearly three quarters of the phytate was detectable in the soluble fiber fraction. The rest of the phytate in the dough was recovered in the supernatant obtained after ethanol precipitation and centrifugation. There is a discrepancy between the content of phytate analyzed in the bread dough and content of phytate obtained by summarizing the amount of phytate analyzed in the soluble fiber and in the supernatant. However, the trend of the figures is the same. As the phytate methodology gives a good reproducibility, this could not explain the discrepancies. We do not have any explanation for this observation, as there are no systematic differences in the figures.

During fermentation the phytate associated to soluble fiber components was broken down, more or less to the same extent as the phytate in the dough. By contrast, the phytate content in the supernatant after alcohol precipitation was constant, regardless of fermentation period or baking. When soluble fiber components were recovered by dialysis, approximately the same amount of phytate was found as by ethanol precipitation and centrifugation.

These findings could be explained in two ways: 1) the main part of phytate is associated to soluble fiber components and will coprecipitate with ethanol together with these components. When the soluble fiber components are recovered with dialysis, the phytate will remain with the soluble fiber components in the sack after dialyzing. Or, 2) the phytate molecules are, to a great extent, complexed in aggregates which will precipitate with the soluble fiber components during ethanol precipitation. These phytate aggregates are too big to pass the dialysis sack used in the dialysis experiment.

Some phytate molecules are not associated with soluble fiber components and/or are small enough to pass through the sack used

TABLE II
Content of Minerals in Bread Dough and in the Different Fiber Fractions
After Fermentation and Breadmaking, and Incubation of the Soluble Fibers^a

Samples	Minerals Associated to Different Fiber Fractions (% of minerals present in dry bread dough) ^b														
	Associated to Insoluble Fiber					Associated to Soluble Fiber (Recovered by Dialysis)					Associated to Soluble Fiber After Additional Incubation (Recovered by Dialysis)				
	Ca	Mg	P	Fe	Zn	Ca	Mg	P	Fe	Zn	Ca	Mg	P	Fe	Zn
A ₁	23	3	4	35	16	61	9	24	29	24	7	0.4	4	16	5
A ₂	22	3	4	35	16	42	6	17	19	16	6	0.2	5	16	0
A ₃	23	3	3	32	13	29	6	14	21	7	9	0.5	6	17	0
A ₄	23	3	4	43	18	25	2	9	19	14	8	0.4	6	21	0
A ₅	24	3	4	33	14	24	5	17	21	7	16	0.8	7	16	0

Minerals in the bread dough
(mg/g dry bread dough)

Ca	0.59
Mg	2.80
P	7.20
Fe	0.081
Zn	0.055

^aMeans of two independent experiments, samples analyzed in duplicate.

^bThe standard deviations were for Ca \pm 4%, Mg \pm 1%, P \pm 2%, Fe \pm 3%, Zn \pm 3%, calculated from duplicate analyses.

TABLE III
Phytate Content During Fermentation and Breadmaking
(mg/g dry matter of the bread dough)^a

Samples	Bread Dough	Insoluble Fiber	Soluble Fiber (Precipitation)	Supernatant	Soluble Fiber (Dialysis)	Soluble Fiber (After Additional Incubation)
A ₁	17.9 ^b	not detectable	13.8	5.9	11.7	7.9
A ₂	16.4	not detectable	9.3	5.3	9.9	6.3
A ₃	11.4	not detectable	4.2	5.3	5.3	3.0
A ₄	9.0	not detectable	4.2	5.5	2.2	1.0
A ₅	7.2	not detectable	2.0	6.6	2.9	1.0

^aThe standard deviation of the method was \pm 1.2 mg/g dry matter, calculated from duplicate analyses. Means of two independent experiments, analyzed in duplicate.

^bThe percent of phytate in the dough was 1.8% (mean of two experiments); the percent phytate if all P in the dough were phytate would be 2.7%, and the percent P as phytate would be 65%.

during dialysis and will not be precipitated with ethanol. A further reduction of the phytate (down to around 5%) was observed with an additional incubation with phytase prepared from wheat. The supernatant from the first centrifugation after enzyme digestion in the fiber assay (including soluble fiber) was incubated for an additional 18 hr at 37°C. But even with such an extremely long incubation time, a complete hydrolysis of phytate was not obtained.

Probably, the most active phytase in the dough is the phytase from yeast. It has been shown by Harland and Harland (1980) that phytate hydrolysis can be increased by increasing the amount of yeast in the dough. In our study, the phytase added for the incubation experiment is, on the other hand, prepared from wheat. The phytate available for this specific phytase might be less and more limited than the phytate available for the phytase from yeast.

Fate of Specific Minerals

The content of selected, specific minerals in the bread dough is shown in Table II. The percentage of these minerals associated with insoluble and soluble fiber components is also shown. Most minerals associated with fiber components occur in the soluble fiber fraction (Frølich and Asp 1980, Schweizer et al 1984, Frølich et al 1984). The minerals behave differently though, both in binding to fiber fractions before fermentation and behavior during fermentation.

Although the amount of ash associated to insoluble fiber components was small and not influenced by fermentation, this fraction contained 35–40% of the iron present in the bread dough, 23% of the calcium, and 16% of the zinc, but only 3–4% of the magnesium and phosphorus. However, no phytate could be detected, which is in accordance with the low amount of phosphorus.

The picture for the minerals associated with soluble fiber components was different: 61% of the calcium, 29% of the iron, 24% of the zinc and phosphorus, but only 9% of the magnesium in the dough was recovered in this fraction. During fermentation minerals associated to this fraction decreased. The content of soluble fiber components during this process was only slightly changed, whereas the phytate decreased. Thus, much of the minerals in the soluble fiber fraction seems to be complexed with phytate and liberated during the hydrolysis of phytate. All the phosphorus recovered in the soluble fiber fraction seems to be present as phytate (0.65% phytate of the bread dough at time zero and 0.20% after 17 hr of fermentation).

Iron

As mentioned earlier, compared with the other minerals, considerably more of the total iron was associated with the insoluble fraction. Iron associated with soluble fiber also behaved differently than other minerals in relation to phytate degradation. The amount of iron associated with this fraction only decreased during the first two hours of fermentation, from 30 to 20% of the total iron in the bread dough, and remained constant at around 20% after that. Even with an additional 18 hr of incubation with phytase, this amount was constant. This could indicate that only 10% of the iron is associated to phytate, whereas up to 60% is associated to fiber components (both insoluble and soluble) not affected by fermentation.

During fermentation there was an elimination of available binding sites caused by the hydrolysis of phytate. A complete hydrolysis of phytate was never obtained. However, Brown et al (1961) stated that only eight of the twelve dissociated protons of phytate are available for metal binding. Most probably, the remaining phytate does not have the ability to bind minerals. This is supported by the fact that zinc, which is known to associate strongly with phytate, was not associated to the remaining phytate after incubation and dialysis. The same was true for magnesium.

This is in contrast to previous reports on iron that stated that iron is mainly present as monoferric phytate (Morris and Ellis 1976). In another paper, however, these authors showed that in enzymatically produced low-phytate bran, monoferric phytate was not present (Morris and Ellis 1980).

Zinc, Magnesium, and Calcium

Zinc and magnesium seemed to be associated with phytate, as they were released from the soluble fiber fraction during fermentation. With the additional phytase incubation, both minerals were totally released. The calcium present in the soluble fiber fraction was decreased from 60 to 25% during the 17 hr of fermentation. With additional incubation, calcium was further released to under 10% of the total calcium present in the original bread dough.

Calcium seemed to be more strongly associated with phytate than, for example, zinc or magnesium. It has been suggested that there is a complexing between calcium, magnesium, and zinc and the phytate molecule (Likuski and Forbes 1965; Oberleas et al 1966a,b; Lease 1967). An explanation for our findings could be that magnesium and zinc are more easily released from this complex than calcium.

In the soluble fiber fraction a small amount of calcium is not released during fermentation. It is recognized that calcium is bound not only to phytate, but also to fiber components, as pectin and other noncellulosic fiber. This could explain the calcium remaining in the soluble fiber fraction. (James 1980).

A reassociation of some of the minerals seems to occur during the breadmaking. This could be due to Maillard reaction products (Johnson et al 1983).

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