Optimal Conditions for Phytate Degradation, Estimation of Phytase Activity, and Localization of Phytate in Barley (Cv. Blenheim)

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Using a multivariate experimental design, optimal conditions for phytate degradation were found to be pH 4.8 and 57 °C in barley flour (cv. Blenheim) and pH 5.2 and 47 °C in a crude extracted phytase from barley. Three methods for measuring phytase activity in raw and hydrothermally processed barley were compared. Incubation at pH 5 and 55 °C for 60 min did not give significantly different results ($p > 0.05$), whereas incubation at pH 5 and 50 °C for 10, 20, 30, and 60 min gave significantly different results ($p < 0.001$) between methods. The change in microstructure of phytate globoids during hydrothermal processing showed that the degradation was highest in the scutellum cells and less in the aleurone layer.

Keywords: *Phytase; phytate; barley; microscopy*

INTRODUCTION

Phytate (*myo*-inositol hexaphosphate) is the principal storage form of phosphorus in plants, particularly in cereal grains and legumes (Maga, 1982). Phytate is a strong chelator of cations and binds minerals such as zinc (Lönnerdal et al., 1988; Sandberg, 1991; Rossander et al., 1992; Sandström and Sandberg, 1992), calcium (Heaney et al., 1991), and iron (Hallberg et al., 1989; Brune et al., 1992; Rossander et al., 1992), making them unavailable for absorption in the human intestine. Cereals or legumes are staple foods in a large part of the world and a major source of minerals, so it is important that the minerals from these products have a high bioavailability. The intake of large amounts of foods rich in phytate may cause several mineral deficiency symptoms (Maga, 1982; Torre et al., 1991). The bioavailability of iron and zinc in the cereals will improve if phytate is degraded (Nävert et al., 1985; Brune et al., 1992), and the removal of phytate from plant foods thus becomes of nutritional significance.

It is possible to degrade phytate in cereals and legumes by means of the naturally occurring enzyme phytase during food processing such as soaking, malting, and bread-making. Lower *myo*-inositol phosphates, inorganic phosphate, and, in some cases, free *myo*-inositol (Cosgrove, 1980) are thus formed. This can also occur in the stomach and small intestine of humans if the dietary phytase is active (Sandberg and Andersson, 1988). To be successful in obtaining enzymatic degradation, it is necessary to know the optimal pH and temperature conditions for phytase. Optimal conditions for extracted barley phytase activity from 4-day-old barley seedlings (Greiner et al., 2000), from malted barley (Lee, 1990), and from green barley malt (Lüers and Silbereisen, 1927) have been reported to be between 40 and 55 °C and pH 5−6. Optimal conditions for phytase activity in extracted phytase from the cereal may not be optimal conditions for phytate degradation in the cereal. The latter is also dependent on accessibility between enzyme and substrate, which in turn is affected by pH and the microstructure of the grain.

The most widely used technique to estimate phytase activity is to measure the release of inorganic phosphorus during incubation of the cereals and legumes as they are (Dagher et al., 1987; Kilmer et al., 1994) or as extracted phytases with added sodium phytate as substrate (Yoshida et al., 1975; Beal and Mehta, 1985; Kikunaga et al., 1991; Eeckhout and Paepe, 1994; Niziolek, 1995; Barrier-Guillot et al., 1996). The estimation of phytase activity by this principle is unspecific, because phosphorus released from phosphorus-containing compounds other than phytate during the incubation is included. Another method for estimation of phytase activity is to incubate the sample and measure the degradation of phytic acid (*myo*-inositol hexaphosphoric acid). Estimation of phytase activity by measurement of degraded phytic acid can also be performed on crude extracted phytases, after removal of *myo*-inositol phosphates in the extract and addition of a specific amount of sodium phytate (Rutgersson et al., 1997). These methods are more specific compared to measurements of released inorganic phosphorus, but they are more time-consuming and require more advanced equipment.

Microscopic studies of the aleurone cells of barley have demonstrated that mature aleurone cells contained organelles, referred to as aleurone grains (Jacobsen et al., 1971; Palmer, 1987). In wheat, these protein bodies contain two inclusions embedded in a protein matrix. Type I inclusions are globoids and contain phytin, whereas type II inclusions are high in niacin (Fulcher et al., 1979). Our previous microscopic studies of the degradation of phytate globoids by processing and enzymes showed that after phytase (*Aspergillus niger*) treatment, these deposits were no longer detected by toluidine blue. Examination of numerous sections re-

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10.1021/jf000525a CCC: $19.00
Published on Web 09/29/2000

Table 1. Barley Raw Material (Batch 1) Incubated as Flour or as Crude Extracted Phytase for 60 min To Find Optimal Conditions for Phytase Activity[a]

	real values		sealed values		flour	extracted phytase
expt	pH	temp (°C)	x_1	x_2	phytase act. (μmol of IP_6/min·g)	phytase act. (μmol of IP_6/min·g)
1	4.0	37.0	−1.0	−1.0	0.122	0.019
2	6.2	37.0	1.0	−1.0	0.071	0.277
3	4.0	60.0	−1.0	1.0	0.088	0.052
4	6.2	60.0	1.0	1.0	0.097	0.044
5	4.0	48.5	−1.0	0.0	0.147	0.152
6	6.2	48.5	1.0	0.0	0.092	0.228
7	5.1	37.0	0.0	−1.0	0.124	0.363
8	5.1	60.0	0.0	1.0	0.163	0.177
9	5.1	48.5	0.0	0.0	0.156	0.456
10	5.1	48.5	0.0	0.0	0.161	0.438
11	5.1	48.5	0.0	0.0	0.157	0.412
12	5.1	55.0	0.0	0.6	0.170	0.418
13	5.2	47.0	0.1	−0.1	0.157	0.437
14	4.8	52.0	−0.3	0.3	0.167	0.390

[a] Values are means of $n = 2$.

vealed that phytate is easy to detect with toluidine blue provided that cell structures are relatively intact (Parkkonen et al., 1997).

The aim of this study was to find optimal temperature and pH conditions for phytate degradation in barley flour and for phytase activity in a crude extracted phytase from barley using a multivariate experimental design. Another aim of this study was to find a reliable, simple, and fast method to measure phytase activity in raw material of barley, processed barley, and barley samples taken out during processing. We therefore compared three methods for estimation of phytase activity in unprocessed and processed barley (cv. Blenheim). Furthermore, the localization of phytate globoids in the barley kernel and the degradation of phytate globoids during hydrothermal processing was studied with fluorescence microscopy to examine how the microstructure of the phytate globoids changes during processing, how this alteration is related to measured phytate degradation during the processing, and to localize the degradation of phytate in the kernel.

MATERIALS AND METHODS

Barley Seeds. Whole barley kernels (cv. Blenheim) from the 1994 crop were provided by Skanska Lantmannen (Sweden) and used throughout the study. Two different batches were used, batches 1 and 2. Batch 1 had a germination index (GI) of ~7.8, and batch 2 had a germination index of 9. A high GI indicates a high enzymatic activity or a high ability to produce enzymes in response to gibberellic acid (Bamforth and Barclay, 1993). The GI was measured by germinating the barley kernels for 2 days in the presence of hydrogen peroxide and it was calculated as

$$\text{GI} = [\text{sum of germinated kernels} \times 10]/[\text{no. of germinated kernels day 1} + (\text{no. of germinated kernels day 2}) \times 2]$$

Batch 1 was used in experiments to find optimal pH and temperature conditions for phytate degradation in barley flour and for extracted phytase from barley. Batch 2 was used for comparing methods to estimate phytase activity and in samples studied by fluorescence microscopy.

Optimal Conditions for Phytate Degradation. To compare optimal conditions for phytate degradation in barley flour and a crude extracted phytase from the seeds, the ground barley raw material (batch 1) and a crude extracted phytase from the same raw material were incubated at various pH values and temperatures for 60 min (Table 1) and the degradation of *myo*-inositol hexaphosphate was determined. *myo*-Inositol hexaphosphate was analyzed in the ground barley raw material before and after incubation by using HPLC as described earlier (Sandberg and Ahderinne, 1986; Sandberg et al., 1989). Duplicate samples (1 g) of the ground barley raw material were incubated in 10 mL of a 0.1 M citrate buffer, the enzymatic activity was stopped after 60 min, the remaining *myo*-inositol phosphates were extracted by adding 0.67 M HCl (30 mL), and the samples were magnetically stirred for 3 h. The remaining *myo*-inositol hexaphosphates were analyzed by using HPLC. Phytase from the seeds was extracted in duplicate by adding 10 volumes of 0.1 M sodium acetate buffer at 4 °C, pH 5.0, according to the method of Konietzny et al. (1995). The samples were magnetically stirred for 2 h at 4 °C and centrifuged at 10000*g* at 4 °C for 30 min. The supernatants were shaken with AG 1-X8 anion resin (Bio-Rad Laboratories, Richmond, CA) for 10 min to remove phosphorus and *myo*-inositol phosphates. Finally, the samples were centrifuged again, and the supernatants were used as enzyme extracts. Phytase activity was measured by incubating the extracted enzyme, sodium phytate, and buffer in equal proportions as was used by Lolas and Markakis (1977). The reaction mixture contained 2 mL of extracted enzyme, 0.8 mL of 15 mM sodium phytate solution (previously adjusted to the incubation pH with 2 M HCl), and 9.2 mL of 0.13 M citrate buffer. The enzymatic activity was stopped after 60 min by adding 4 mL of 2 M HCl. The samples were evaporated to dryness and analyzed for *myo*-inositol hexaphosphate by using HPLC as described earlier (Sandberg and Ahderinne, 1986; Sandberg et al., 1989).

The first 11 experiments were carried out as a central composite face centered design (CCF; Umetri, 1992−1997). Experiments 1−8 in Table 1 represent a full factorial design in two levels, and experiments 9−11 were replicates carried out at the center point. Experiment 12 was carried out at pH 5.1 and 55 °C, which are optimal conditions for wheat phytase (Peers, 1953). Two verification experiments were carried out in the experimental domain (experiments 13 and 14) to study the reliability of the mathematical model fitted to the experiments in the CCF design. The pH values and the temperatures used in the 14 experiments are presented in Table 1 as real values and scaled values (see Statistical Evaluation).

Estimation of Phytase Activity. *Processing of the Seeds.* To compare three methods for estimation of phytase activity in raw barley and processed barley, samples with various phytic acid contents and various expected phytase activities were used as test material. These barley samples were produced in a study published earlier (Bergman et al., 1999). The barley was hydrothermally processed at different temperatures in lactic acid solutions of various concentrations as described earlier (Bergman et al., 1999) and is here described schematically in Figure 1. The hydrothermal process presented in Figure 1 is a modernization of an old hydrothermal process (Meyer-Renschhausen, 1991) that was used to facilitate de-

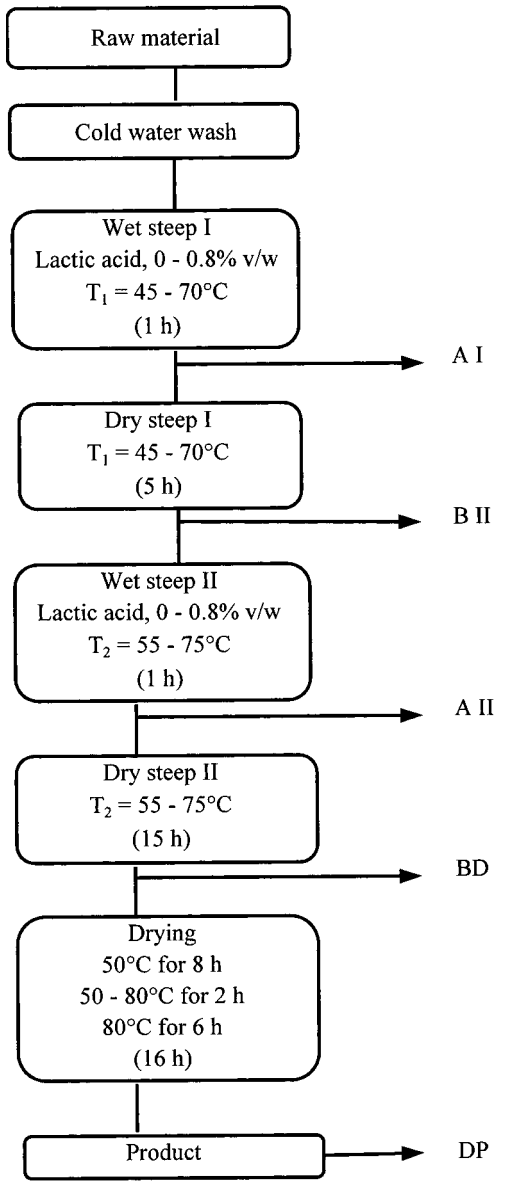

Figure 1. Flowchart of the hydrothermal process. Samples were taken out after the first wet steep (AI), before the second wet steep (BII), after the second wet steep (AII), before drying (BD), and as dried end product (DP).

hulling of cereals before modern milling techniques prevailed. This process resembles the parboiling process that is now used for rice. The temperature during processing was varied between 48 and 65 °C, and the concentration of lactic acid solution was varied between 0.4 and 0.8% v/w, according to Tables 2 and 3. For phytase activity measurements, samples were taken out after the first wet steep (AI in Figure 1, experiments 1−5 in Table 2) or after wet steep two (AII in Figure 1, experiments 6−11 in Tables 2 and 3). Experiment 12 included soaking for 1 h and then a dry steep for 6 h at the same temperature as was used during the wet steep (experiment 12, Table 3). The seeds were freeze-dried and stored at −20 °C until analyzed for phytase activity.

Measurements of Phytase Activity. Approximately 30 g of whole barley kernels was thawed and ground for 30 s with a coffee grinder (Philips, HR 2185, Netherlands), and 0.4 g was analyzed in duplicate for dry matter (dm) by using a moisture balance (Precisa, HA 300, Switzerland) before phytase activity was measured. More than 85% of the barley flour had a particle size of <1 mm.

Phytase activity was estimated by using the three different methods described below. Phytase activities were estimated at pH 5, which is between the optimal pH for phytate degradation in barley flour (pH 4.8) and that for the crude extracted phytase (pH 5.2) found in this study. The temperatures used during the phytase activity estimations were 55 and 50 °C; 55 °C is close to the optimal temperature for phytate degradation in barley flour, and this temperature has been used previously in phytase activity estimations in cereals (Peers, 1953; Tomlinson and Ballou, 1962; Fretzdorff and Weipert, 1986). The temperature of 50 °C was chosen because it is between the optimal temperature for phytate degradation in barley flour (57 °C) and in crude extracted phytase from barley (47 °C) found in this study, and this temperature has also been used by others (Frölich et al., 1986).

Method 1: Determination of Released Phosphorus during Incubation of Barley Flour. Free inorganic phosphorus was measured spectrophotometrically, before and after incubation of the samples, according to the method of Fiske and Subbarow (1925) with some modifications. Eight milliliters of a 3% trichloroacetic acid and 4 mL of a 20 mM citrate buffer, pH 5, were added to duplicate samples (0.4 g). Twelve milliliters of deionized water was added after 5 min. The samples were centrifuged, and 0.5−1.5 mL of the supernatants was mixed with 4.0 mL of a 2.5% sulfuric acid, 1 mL of a 50:50 mixture of ammoniummolybdate and dimethyl sulfoxide, and 0.25 mL of aminonaphtholsulfonic acid. Inorganic phosphorus was determined spectrophotometrically (Hitachi, model 101). Duplicate samples (0.4 g) from the same material were soaked in 4 mL of a 20 mM citrate buffer at pH 5 and 55 °C for 60 min (experiments 1−10, Table 2) or at pH 5 and 50 °C for 10, 20, 30, and 60 min (experiments 11 and 12, Table 3). Eight milliliters of a 3% trichloroacetic acid was added to stop enzymatic activity, 12 mL of deionized water was added after 5 min, and free inorganic phosphorus was measured as above. Phytase activity was calculated as micromoles of released inorganic phosphorus per minute and gram dm and related to the phytase activity in the raw material as percentage activity.

Method 2: Determination of myo-Inositol Hexaphosphate Degradation during Incubation of Barley Flour. myo-Inositol hexaphosphate was analyzed in the ground barley samples, before and after incubation. Duplicate samples (0.5 g) of ground raw material or freeze-dried, ground processed seeds were extracted with 0.5 M HCl (20 mL) for 3 h. Duplicate samples (1 g) of the same material were incubated in 10 mL of a 20 mM citrate buffer at pH 5 and 55 °C for 60 min (experiments 1−10, Table 2) or at pH 5 and 50 °C for 10, 20, 30, and 60 min (experiments 11 and 12, Table 3). The enzymatic activity was stopped, and the remaining *myo*-inositol phosphates were extracted by adding 0.67 M HCl (30 mL). The samples were magnetically stirred for 3 h and then analyzed for *myo*-inositol phosphates by HPLC as described earlier (Sandberg and Ahderinne 1986; Sandberg et al., 1989). Phytase activity was calculated as micromoles of degraded *myo*-inositol hexaphosphate per minute and gram dm and related to the phytase activity in the raw material as percentage activity.

Method 3: Incubation of Extracted Phytase with Added Sodium Phytate as Substrate. Phytase from the ground seeds was extracted in duplicate by adding 10 volumes of 0.1 M sodium acetate buffer at 4 °C, pH 5.0, according to Konietzny et al. (1995). The samples were magnetically stirred for 2 h at 4 °C and centrifuged at 10000*g* at 4 °C for 30 min. The supernatants were shaken with AG 1-X8 anion resin (Bio-Rad Laboratories) for 10 min to remove phosphorus and *myo*-inositol phosphates. Finally, the samples were centrifuged again, and the supernatants were used as enzyme extracts. Phytase activity was measured by incubating the crude extracted enzyme with sodium phytate at pH 5 and 55 °C for 60 min (experiments 1−10, Table 2) or at pH 5 and 50 °C for 10, 20, 30, and 60 min (experiments 11 and 12, Table 3). The reaction mixture had the same proportions of extracted enzyme, sodium phytate, and buffer as were used by Lolas and Markakis (1977). The total volume of the reaction mixture was 12 mL, consisting of 2 mL of extracted enzyme, 0.8 mL of sodium phytate solution (15 μmol/mL) previously adjusted to the same pH as that of the citrate buffer with 2 M HCl, and

Table 2. Phytase Activity Measured by Incubating Ground Samples (Methods 1 and 2) or Extracted Phytase (Method 3) for 60 min at pH 5 and 55 °C[a]

				method 1		method 2		method 3	
expt	T_1[b] (°C)	T_2[b] (°C)	C[b] (%)	phytase act.[c]	% phytase act.[d]	phytase act.[c]	% phytase act.[d]	phytase act.[c]	% phytase act.[d]
raw material				0.98 ± 0.025	100.0	0.23 ± 0.008	100.0	0.43 ± 0.017	100.0
1	60		0.6	0.46 ±0.008	47.5	0.12 ± 0.004	54.1	0.19 ± 0.008	42.9
2	65		0.4	0.18 ± 0.011	18.1	0.05 ± 0.003	22.5	0.01 ± 0.011	2.2
3	55		0.4	0.49 ± 0.010	49.8	0.15 ± 0.002	64.5	0.17 ± 0.001	40.0
4	55		0.4	0.47 ± 0.038	48.2	0.15 ± 0.010	64.1	0.24 ± 0.014	55.0
5	55		0.4	0.58 ± 0.026	59.1	0.14 ±0.003	59.1	0.21 ± 0.010	49.5
6	60	60	0.6	0.13 ± 0.002	13.3	0.02 ± 0.007	8.1	0.03 ± 0.003	6.6
7	65	65	0.4	0.11 ± 0.010	11.2	0.00 ± 0.002	0.3	0.03 ± 0.009	6.1
8	55	65	0.4	0.10 ± 0.003	10.5	0.02 ± 0.002	10.8	0.01 ± 0.006	2.0
9	55	55	0.4	0.22 ± 0.074	22.4	0.08 ± 0.005	34.6	0.12 ± 0.000	27.7
10	55	65	0.4	0.19 ± 0.011	19.6	0.04 ± 0.001	18.6	0.07 ± 0.000	16.8

[a] Values are mean ± SD for $n = 2$. [b] T_1 and T_2, temperatures in the first and second soaking steps, respectively. C, lactic acid concentration during soaking. [c] Phytase activity calculated as released μmol of P/min·g of dm for method 1 and as degraded μmol of IP_6/min·g of dm for methods 2 and 3. [d] Phytase activity related to the phytase activity in the raw material. These values were used in the statistical comparison of the methods.

Table 3. Phytase Activity Measured by Incubating Ground Samples (Methods 1 and 2) or Extracted Phytase (Method 3) for 10−60 min at pH 5 and 50 °C[a]

	time	method 1		method 2		method 3	
expt	(min)	phytase act.[b]	% phytase act.[c]	phytase act.[b]	% phytase act.[c]	phytase act.[b]	% phytase act.[c]
raw material	10	0.81 ± 0.037	100.0	0.68 ± 0.057	100.0	1.02 ± 0.026	100.0
	20	0.89 ± 0.005	100.0	0.49 ± 0.039	100.0	0.74 ± 0.029	100.0
	30	0.86 ± 0.003	100.0	0.36 ± 0.016	100.0	0.61 ± 0.017	100.0
	60	0.64 ± 0.022	100.0	0.20 ± 0.008	100.0	0.47 ± 0.000	100.0
11[d]	10	0.66 ± 0.181	83.7	0.34 ± 0.010	49.5	0.92 ± 0.018	90.2
	20	0.63 ± 0.022	70.6	0.24 ± 0.002	48.6	0.64 ± 0.035	86.5
	30	0.49 ± 0.009	56.8	0.17 ± 0.001	48.6	0.52 ± 0.033	85.2
	60	0.36 ± 0.018	56.3	0.09 ± 0.001	47.4	0.41 ± 0.007	87.2
12[e]	10	0.95 ± 0.188	118.1	0.41 ± 0.004	60.3	0.85 ± 0.002	83.3
	20	0.69 ± 0.017	77.8	0.29 ± 0.002	58.6	0.60 ± 0.015	81.1
	30	0.58 ± 0.039	67.2	0.20 ± 0.002	56.8	0.51 ± 0.014	83.6
	60	0.40 ± 0.017	62.4	0.11 ± 0.001	54.3	0.38 ± 0.009	80.9

[a] Values are mean ± SD for $n = 2$. [b] Phytase activity calculated as released μmol of P/min·g of dm for method 1 and as degraded μmol of IP_6/min·g of dm for methods 2 and 3. [c] Phytase activity related to the phytase activity in the raw material. These values were used in the statistical comparison of the methods. [d] 48 °C and 0.8% lactic acid solution were used in the two soaking steps. [e] 48 °C and 0.8% lactic acid solution were used in the soaking step.

9.2 mL of citrate buffer (0.13 M). The final concentrations of buffer and phytate were 0.1 M and 1 mM, respectively. After the incubation, 4 mL of 2 M HCl was added to stop enzymatic activity. The samples were evaporated to dryness and analyzed for *myo*-inositol phosphates as described earlier (Sandberg and Ahderinne, 1986; Sandberg et al., 1989). Phytase activity was calculated as micromoles of degraded *myo*-inositol hexaphosphate per minute and gram dm and related to the phytase activity in the raw material as percentage activity.

Statistical Evaluation. The results of the 12 experiments to find optimal conditions for phytase activity by incubating the raw material (batch 1) as flour (method 2) or extracted phytase (method 3) at various pH values and temperatures were statistically evaluated by multiple linear regression (MLR) using the computer program Modde 3.0 (Umetri, 1992−1997). A CCF design makes it possible to approximate the measured data (y_{obsd}) with a response surface model expressed in scaled values:

$$y_{obsd} = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 + \epsilon \quad (1)$$

x_1 is the scaled value of the pH [$x_1 = (\text{pH} - 5.1)/1.1$], and x_2 is the scaled value of the temperature [$x_2 = (T - 48.5)/11.5$]. The coefficient b_0 is a constant, b_1 and b_2 are the main effects of x_1 and x_2, respectively, b_{12} shows the interaction between the variables, and the square coefficients b_{11} and b_{22} will tell if any of the variables has a maximum/minimum in the experimental domain. The difference between the experimental data (y_{obsd}) and the model (y_{calcd}) gives the residual (ϵ). The replicates at the center point make it possible to estimate the pure errors of the analyses, which is used to predict if the models give significant lack of fit (Carlsson, 1992). R^2 and Q^2 were calculated for each response. R^2 is the fraction of variation of the response explained by the model, and Q^2 is the fraction of variation of the response that can be predicted by the model (Umetri, 1992−1997). R^2 is an overestimated and Q^2 an underestimated measure of the goodness of fit of the model. Both R^2 and Q^2 should be as close to 100% as possible, but if conclusions are to be drawn from the model, Q^2 should exceed 50% and if R^2 and Q^2 are >90% the model is considered to be excellent.

The results obtained by incubating the raw material and processed barley samples (batch 2) at pH 5 and 55 or 50 °C and measuring phytase activity using methods 1−3 were evaluated by fitting linear models to the results by the General Linear Model procedure (GLM) using the computer program SAS (Statistical Analysis System, version 8). The terms included in the models were the methods, the experiments, the incubation times (only for experiments 11 and 12), and all of the interaction terms of these terms. Duncan's multiple-range test (Duncan, 1975) was used to check if the different methods, experiments, and incubation times gave significantly different relative phytase activities on the 5% level ($p < 0.05$).

Fluorescence Microscopy. Barley samples examined with fluorescence microscopy to study where the phytate globoids in the barley kernels are situated were taken out after the first wet steep (AI), before the second wet steep (BII), after the second wet steep (AII), before drying (BD), and as dried end products (DP). Samples from experiments 13−21 were studied microscopically and analyzed for phytate content as described earlier (Sandberg and Ahderinne, 1986; Sandberg et al., 1989). Samples from experiments 17−21 were collected,

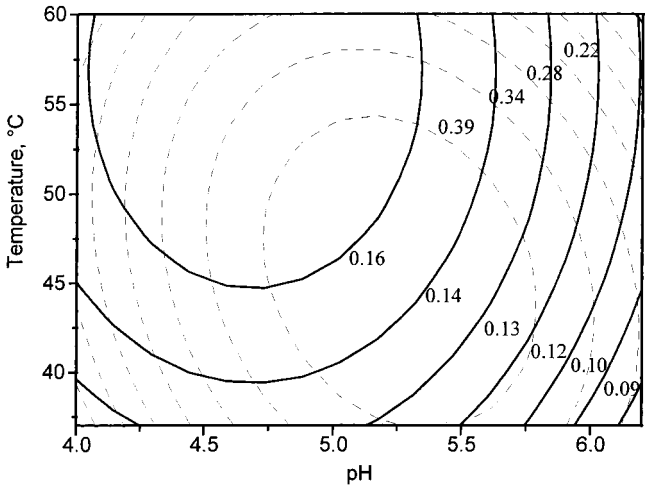

Figure 2. Response surface plots of phytase activities in barley flour (—) and extracted phytase (- - -). Values are μmol of degraded IP_6/min·g of dm.

cooled, and fixed in glutaraldehyde (pH 7.0) for microscopic studies or freeze-dried for measurement of the phytate content.

Barley grains and corresponding hydrothermally treated grains (Figure 1) were fixed in glutaraldehyde (pH 7.0) (Parkkonen et al., 1994), washed with water, and dehydrated in a series of ethanol. Samples were infiltrated and polymerized with Historesin Embedding Kit (Jung, Germany). Sections were cut 5 μm thick with a microtome (Leica Jung RM2055, Germany) and placed on a drop of water on an objective slide. After drying, the plastic sections were firmly adhered to a slide. Phytate appeared as violet deposits after staining with 0.05% toluidine blue (Fulcher et al., 1981; Parkkonen et al., 1997). After staining, the sections were covered with a cover glass and examined and photographed with an Olympus BH-2 microscope (Japan). The photographs were obtained on Kodak 100 ASA film. Samples examined by fluorescence microscopy are shown in Table 4. Ten kernels from each sample were examined.

RESULTS

Optimal Conditions for Phytate Degradation. The results of incubation of the barley raw material (batch 1) as flour to find optimal conditions for phytate degradation are shown in Table 1. The results of the 11 experiments in the CCF design plus experiment 12 were statistically evaluated by MLR by using the computer program Modde 3.0 (Umetri, 1992−1997). The normal probability plot showed experiment 3 to be an outlier, and that experiment was thus excluded from the calculations. The achieved mathematical model expressed in scaled values was

$$y_{\text{calcd}}\ (\mu\text{mol of IP}_6\ \text{degraded/min·g of dm}) = 0.16 - 0.03x_1 + 0.02x_2 - 0.00x_1x_2 - 0.03{x_1}^2 - 0.01{x_2}^2 \quad (2)$$

Factors that had a significant influence ($p < 0.02$) on the phytase activity were the pH (x_1), the temperature (x_2), and the square terms of pH and temperature (x_1^2 and x_2^2, respectively). As the interaction term did not have a significant effect on the phytase activity, this term was removed from the model. The model showed no significant lack of fit (see ANOVA table, Table 5), R^2 and Q^2 were 99 and 96%, respectively, which makes it possible to draw conclusions from the model. As can be seen from the model and the response surface plot (eq 2 and Figure 2), there was an optimum for phytate degradation in barley flour in the experimental domain

Table 4. Samples Studied with Fluorescence Microscopy and Analyzed for Phytate Content[a]

expt ($T_1/T_2/C$)[b]	AI[c]	BII[c]	AII[c]	BD[c]	DP[c]
raw material					large amount in aleurone and germ (IP_6 = 13.88)
13 (55/55/0.0)	not studied	not studied	not studied	not studied	large amount in aleurone and germ (IP_6 = 5.51)
14 (55/55/0.1)	not studied	not studied	not studied	not studied	large amount in aleurone and germ (IP_6 = 4.84)
15 (55/55/0.6)	not studied	not studied	not studied	not studied	very low amount, only in aleurone (IP_6 = 1.86)
16 (70/70/0.6)	not studied	not studied	not studied	not studied	large amount in aleurone, low amount in germ (IP_6 = 8.75)
17 (70/70/0.6)	not studied	not studied	some phytate in aleurone and germ (IP_6 = 8.90)	some phytate in aleurone and germ (IP_6 = 7.99)	some phytate in aleurone and germ (IP_6 = 8.20)
18 (45/65/0.4)	some aleurone cells full, some empty, less in germ (IP_6 = 9.50)	decreased amount in germ (IP_6 = 10.43)	decreased amount in some aleurone cells, less in germ (IP_6 = 6.71)	large amount in aleurone and germ (IP_6 = 5.96)	some phytate in aleurone and germ (IP_6 = 6.05)
19 (55/65/0.4)	still much left in aleurone cells, less in germ, spreading of globoids (IP_6 = 10.19)	decreased amount in germ, phytate globoids in aleurone cells lost their shape (IP_6 = 6.50)	decreased amount in aleurone, more reduced in germ, size of globoids was decreased (IP_6 = 6.25)	some in aleurone, some in germ (IP_6 = 5.03)	no globoids left (IP_6 = 4.91)
20 (55/75/0.4)	still much left in aleurone cells, less in germ, spreading of globoids (IP_6 = 9.33)	not studied (IP_6 = 6.36)	larger amount in aleurone than in germ (IP_6 = 5.89)	larger amount in aleurone layer than in germ (IP_6 = 6.18)	phytate in some aleurone cells, some empty, a few in germ (IP_6 = 4.72)
21 (55/65/0.8)	small amount in aleurone cells, less in germ (IP_6 = 10.11)	not studied (IP_6 = 5.26)	some in the aleurone cells and some in germ (IP_6 = 4.62)	very few in aleurone cells, none in germ (IP_6 = 2.83)	no globoids left (IP_6 = 3.22)

[a] IP_6 values are μmol/g of dm, means of $n = 2$. [b] T_1 and T_2, temperatures in the first and second soaking steps, respectively. C, lactic acid concentration during soaking. [c] After first wet steep (AI), before second wet steep (BII), after second wet steep (AII), before drying (BD), and dried end product (DP).

Table 5. Analysis of Variance for Phytase Activity Results of Experiments To Find Optimal Conditions for Phytase in Barley Flour and Extracted Phytase from Barley

	flour		extracted phytase	
	DF[a]	SS[b]	DF[a]	SS[b]
total	11	0.205	11	1.033
constant	1	0.194	1	0.743
total corrected	10	0.011	10	0.29
regression	4	0.011[c]	5	0.286[c]
residual	6	0.0001	5	0.004
lack of fit (model error)	4	0.0001	3	0.003
pure error (replicant error)	2	0.00001	2	0.001
% variability explained (R^2)		98.0		98.7

[a] DF, degrees of freedom. [b] SS, sum of squares. [c] Significant at 1% level, $p < 0.001$.

Table 6. Predicted and Observed Phytase Activities for the Verification Experiments[a]

			flour		extracted phytase	
expt	pH	temp (°C)	predicted	observed	predicted	observed
13	5.2	47	0.15 ± 0.006	0.16 ± 0.002	0.45 ± 0.033	0.44 ± 0.014
14	4.8	52	0.17 ± 0.005	0.17 ± 0.000	0.39 ± 0.033	0.39 ± 0.036

[a] Values are μmol of degraded IP_6/min·g of dm and are given as mean ± SD for duplicate samples.

at pH 4.8 and 57 °C. Evaluation of the 11 results from experiments in the CCF design plus experiment 12, when extracted phytase from the raw material (batch 1) was incubated to find optimal conditions for phytase activity, gave the following mathematical model expressed in scaled values:

$$y_{calcd}\ (\mu\text{mol of IP}_6\ \text{degraded/min·g of dm}) = 0.44 + 0.06x_1 - 0.04x_2 - 0.07x_1x_2 - 0.25x_1^2 - 0.10x_2^2 \quad (3)$$

Experiment 8 was shown to be an outlier in the normal probability plot and was thus excluded from the calculations. All factors in the model had a significant influence ($p < 0.02$) on the phytase activity. The model showed no significant lack of fit (see ANOVA table, Table 5), the R^2 was 99% and the Q^2 was 88%, and conclusions can thus be drawn from the model. As can be seen from the model and the response surface plot (eq 3, Figure 2), there was an optimum for phytase activity in the barley extract in the experimental domain at pH 5.2 and 47 °C. Two verification experiments were performed within the experimental domain (experiments 13 and 14, Tables 1 and 6). Predicted and observed values for these experiments are shown in Table 6. All observed values were reasonably in accordance with the predicted values, which further proves the mathematical models to be correct.

Phytase Activity Measurements. Results from experiments in which ground freeze-dried barley or extracted phytase from barley was incubated at pH 5 and 55 °C are shown in Table 2. As can be seen, the three methods gave similar relative phytase activities (percent phytase activity) for the various samples. When the results were statistically evaluated, the method used did not have a significant ($p > 0.05$) effect on the results. However, in most cases method 2 gave the highest relative phytase activities, whereas method 3 gave the lowest relative phytase activities in most cases.

Results from experiments in which ground, freeze-dried barley or extracted phytase from barley was incubated at pH 5 and 50 °C for 10, 20, 30, and 60 min are shown in Table 3. Statistical analysis showed that the method and the incubation time had significant effects ($p < 0.001$ and $p < 0.05$, respectively) on the results (percent phytase activity). The interaction term between method and time had also a significant effect ($p < 0.05$). When a linear model including these three terms was fitted to the results, the model gave an R^2 of 88% and the model was significant on the 1% level ($p < 0.001$). Duncan's multiple-range test (Duncan, 1975) showed that all three methods gave significantly different phytase activities ($p < 0.05$). This test also showed that an incubation time of 10 min gave a significantly higher relative phytase activity ($p < 0.05$) than incubation for 30 or 60 min. In this case, method 3 gave the highest relative phytase activities when the samples were incubated for 30 or 60 min, whereas method 2 gave the lowest relative phytase activities for all incubation times.

Fluorescence Microscopy. The results of the microscopic studies are shown in Table 4, and micrographs of the raw material and samples taken out during processing of experiment 19 are shown in Figure 3. The amounts of phytate as *myo*-inositol hexaphosphate (IP_6) are also shown in Table 4. In most samples phytate globoids were found both in the aleurone layer and in the scutellum cells in the germ. The number of phytate globoids present in the kernels was very much dependent on the lactic acid concentration and the temperature during processing. The phytate globoids were most degraded when barley kernels were treated with 0.4−0.8% (v/w) lactic acid concentration at 55−65 °C (experiments 15, 19, and 21), which is in good agreement with the measured phytate levels. Experiment 19 showed a typical phytate degradation behavior (Table 4 and Figure 3). After the first steeping step, the number of phytate globoids in the germ was decreased, and this was also seen in other experiments independent of the treatment (experiments 18−21). After the second steeping step, the appearance of the globoids changed. In experiment 19 the size of the globoids was decreased, whereas, in some experiments, the globoids were swollen. The greatest change in the number of stained globoids occurred during drying. In the end product from experiment 19, no phytate globoids were seen.

DISCUSSION

The optimal conditions we found for phytate degradation in the extracted phytase, pH 5.2 and 47 °C, are in good agreement with the results that have been found by others for phytase activity in extracts from green barley malt (Lüers and Silbereisen, 1927), from malted barley (Lee, 1990), and from 4-day-old barley seedlings (Greiner et al., 2000). Lüers and Silbereisen (1927) found optimal conditions for phytase activity in green barley malt to be pH 5.2 and 48 °C, and Lee (1990) found pH 5.0 and 40 °C to optimal in malted barley. Greiner et al. (2000) purified two phytate-degrading enzymes from 4-day-old barley seedlings (P_1 and P_2). Optimal conditions for phytate degradation by these enzymes were pH 5.0 and 45 °C for P_1 and pH 6.0 and 55 °C for P_2. The optimal conditions we found for phytate degradation in barley flour, pH 4.8 and 57 °C, are close to the conditions found to be optimal for phytate degradation in wholemeal wheat [pH 5.1 and 55 °C (Peers, 1953)]. The difference between optimal conditions for phytase activity in extracted phytase from barley and phytate degradation in barley flour may be due to the

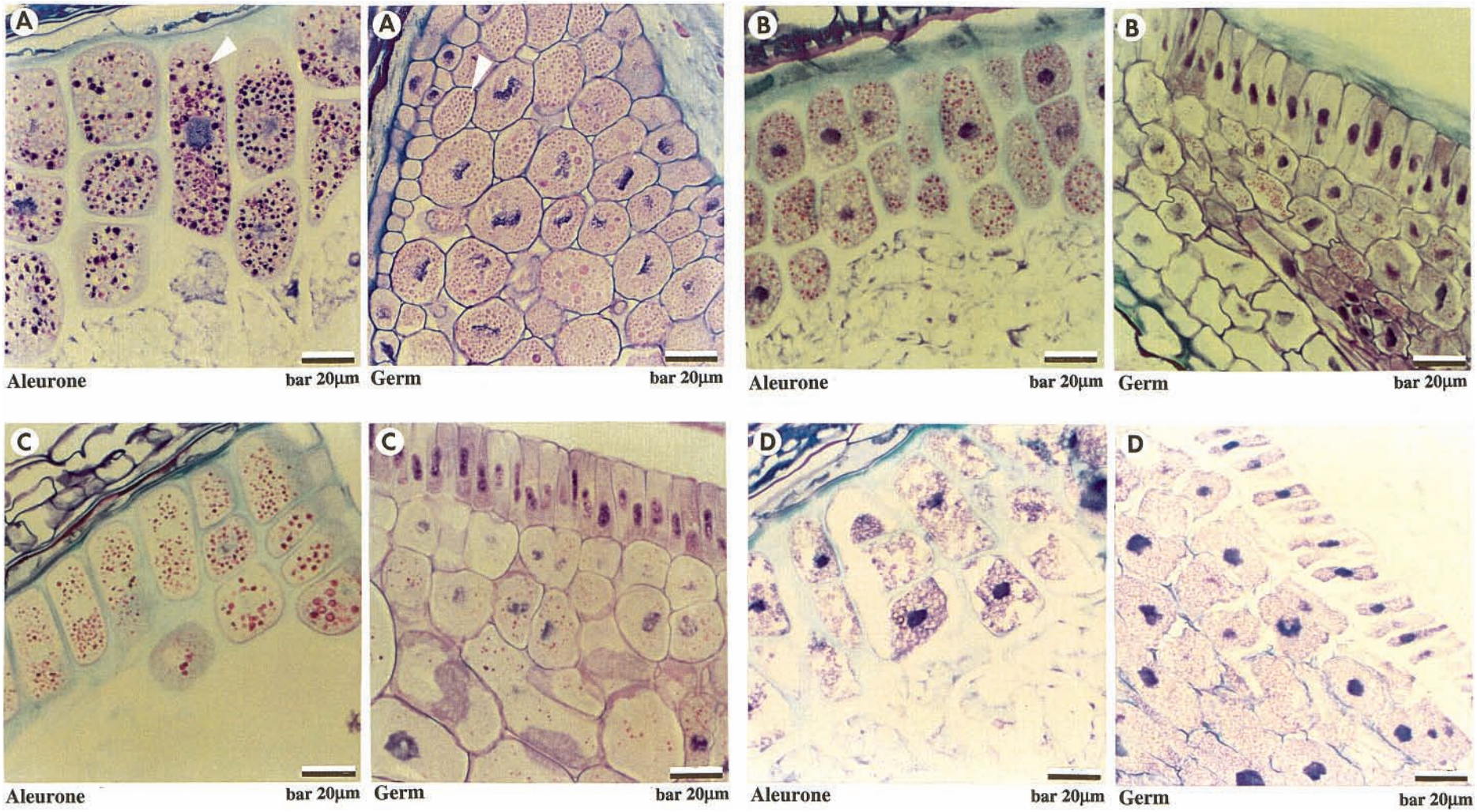

Figure 3. Embedded sections of aleurone layer and scutellum of barley raw material (A) and hydrothermally treated samples from experiment 19 taken out after the first (B) and second wet steeps (C) and after drying (D). The arrow in (A) indicates a phytate globoid.

accessibility between enzyme and the substrate and also due to differences in thermal stability between the phytase in the flour and the extracted phytase. Peers (1953) showed that phytase in wholemeal wheat was more heat-stable than extracted phytase from the wheat. Phytase probably starts to deactivate at a temperature of 45−55 °C (Peers, 1953; Lee, 1990; Greiner et al., 2000). A lower pH and a higher temperature for the incubation of barley flour might favor the contact between the enzyme and its substrate due to microstructural changes and the activity of proteases with a low pH-optimum (Hoseney, 1986). For the extracted phytase, a temperature >50 °C may be negative because of thermal deactivation, whereas the positive effects of a higher temperature outweigh the negative effects of phytase deactivation for phytate degradation in barley flour.

The knowledge of optimal conditions for phytase activity in barley (cv. Blenheim) found in this study could be used during processing, such as malting with subsequent soaking, parboiling, or bread-making to degrade phytate during these processes. If phytate is degraded in cereals during processing, mineral bioavailability increases (Nävert et al., 1985; Brune et al., 1992; Larsson et al., 1996). The knowledge obtained can be used for the production of cereal-based infant formulas, children's food products, breakfast cereals, and breads with a high mineral bioavailability.

The three methods for estimating phytase activity in raw material of barley and in processed barley did not give significantly different results between methods when the incubation temperature was 55 °C ($p > 0.05$), but the methods did give significantly different results when the incubation temperature was 50 °C ($p < 0.001$). When 50 °C was used during incubation, method 3 gave the highest relative phytase activities for the samples when the incubation time was 30 or 60 min. This suggests that method 3 is the preferable method and that the incubation temperature should not exceed 50 °C to achieve a true value of phytase enzymes in a sample that could be active if substrate inhibitors and lower *myo*-inositol phosphates are removed. Method 3 is also preferred when the samples have a low phytate content because in this method phytate is added as substrate, whereas in methods 1 and 2 no phytate is added and possible product inhibitors are not removed. It may be possible that the high activities achieved with method 3 could also be achieved with method 2 if phytate is added at the same level as is used in method 3. In some cases it may be more relevant to use method 2, that is, to estimate how active the phytase enzymes will be during processing such as soaking or hydrothermal processing. Incubation at 57 °C seems most appropriate in that case, because this was the optimal temperature for phytase activity in barley, together with an incubation time of 10−60 min because the time did not have a significant influence on the relative phytase activity. Method 2 could be useful in finding barley cultivars with a high phytase activity suitable for hydrothermal processing to degrade phytate. Malting has been shown to increase phytase activity but not to extensively degrade phytate (Lee, 1990). Method 2 could be used to measure the increase of phytase activity during malting of barley to decide when the phytase activity is high enough to degrade phytate extensively during subsequent soaking at the optimal conditions found in this study. Method 3 would probably be most suitable to decide how much phytase enzyme the malt contains that could be active in the digestive tract on other phytate sources.

The fluorescence microscopy results show that the phytate globoids in barley kernels are situated in the aleurone layer and in the scutellum cells in the germ and that the degradation of phytate globoids during hydrothermal processing is highest in the scutellum cells. This is in good agreement with Peers (1953), who found the phytase activity in wheat to be highest in scutellum. When barley kernels were studied with fluorescence microscopy, there were no visible phytate globoids in the dried end products of experiments 19 and 21 (Table 4, DP; Figure 3, D), whereas there were still some but very few globoids left in the dried end product of experiment 15. However, the dried end product of experiment 15 had the lowest measured content of phytate. Processing of cereals may alter the phytate globoid structure to such an extent that its stainability for microscopic analysis is prevented. It appears that as soon as the sample is mechanically or thermally treated, stainable phytate deposits are no longer detectable (Parkkonen et al., 1997). It seems this is the case during the drying step in the hydrothermal process as the microscopic pictures show that the greatest change in the number of phytate globoids occurs during drying, whereas when the phytate content was measured, no such reduction was found.

ABBREVIATIONS USED

CCF, central composite face centered design; cv., cultivar; dm, dry matter; MLR, multiple linear regression.

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Received for review April 26, 2000. Revised manuscript received July 18, 2000. Accepted July 18, 2000. This study has been financially supported by the Nordic Industrial Foundation, Oy Lahden Polttimo AB (Finland), Semper AB (Sweden), Skanska Lantmannen's Research Foundation (Sweden), and Nutek (Sweden).

JF000525A