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Changes of Folates, Dietary Fiber, and Proteins in Wheat As Affected by Germination

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Wheat kernels of the cultivar 'Tommi' were germinated for up to 168 h at 15, 20, 25, or 30 °C. Samples were taken at different stages of germination and were analyzed for the quantitative protein composition using an extraction/HPLC method, for folate vitamers using a stable isotope dilution assay, and for soluble, insoluble, and total dietary fiber using a gravimetric method. Gluten proteins were substantially degraded during germination. During the first stages of germination the degradation of glutenins was predominant, whereas longer germination times were required to degrade gliadins. The optimal temperature for gliadin degradation was 20 °C, and that for glutenin degradation was 25 °C. ω5- and ω1,2-gliadins were less sensitive to proteolytic degradation than α- and γ-gliadins, and LMW subunits of glutenin were less sensitive than HMW subunits. During germination a time- and temperature-dependent increase of total folate occurred. A maximum 3.6-fold concentration was obtained after 102 h of germination at 20 and 25 °C including 5-methyltetrafolate as the major vitamer. The concentration of dietary fiber remained constant or decreased during the first 96 h of germination. Prolonged germination times of up to 168 h led to a substantial increase of total dietary fiber and to a strong increase of the soluble dietary fiber by a factor of 3, whereas the insoluble fiber decreased by 50%.

KEYWORDS: Wheat; germination; gluten proteins; dietary fiber; folic acid

INTRODUCTION

In many regions of the world wheat is a staple food due to its significant content of starch and proteins. Additionally, whole wheat is an important source of dietary fiber, the consumption of which is reputed to lower the risk of colon cancer (*1*), diabetes mellitus (*2*), and cardiovascular disease (*3*). With regard to micronutrients, the average daily consumption of 200 g of bread covers only about 12−31% of the daily requirements of important vitamins such as riboflavin or thiamin (*4*), respectively. In particular, the vitamins of the folate group have gained increasing public awareness as in Europe the usual diet meets only about 50% of the daily requirement and an adequate folate intake is associated with a lower incidence of neural tube defects, cardiovascular disease, colon cancer, and Alzheimer's disease (*5*). Therefore, folate fortification has become mandatory in several countries, particularly on the American continent, and currently is discussed in the European Union. In contrast to fortification, a natural way to increase vitamin levels is germination of plant seeds and has been applied for several decades. In this way, contents of vitamins such as riboflavin, thiamin, biotin, pantothenic acid, tocopherols, and folates have been reported to amount to 1.5−3.8 times those in ungerminated seeds (*6*, *7*). Similarly, recent evidence indicated a folate increase to 350% during germination of rye (*8*). The content of soluble and insoluble dietary fibers in wheat was found to increase by 25% after 4 days of germination (*9*).

In contrast to the increase in bioactive compounds, the baking performance of wheat dramatically decreases during germination (*10*). This is due to an increase of enzymatic activities, for example, protease and amylase leading to the degradation of proteins and starch to provide the developing plant with nutrients. Therefore, the use of germinated wheat for baking has been restricted up to now.

With regard to the substantial differences in how wheat was germinated and the bioactive compounds and proteins were analyzed in previous studies, the need for systematic investigations including an array of different germination conditions and application of accurate quantitative methods is obvious. As folate analysis, in particular, is very challenging and the existing folate data might not be accurate, newly developed methods such as stable isotope dilution assays (SIDA) (*11*) would allow more

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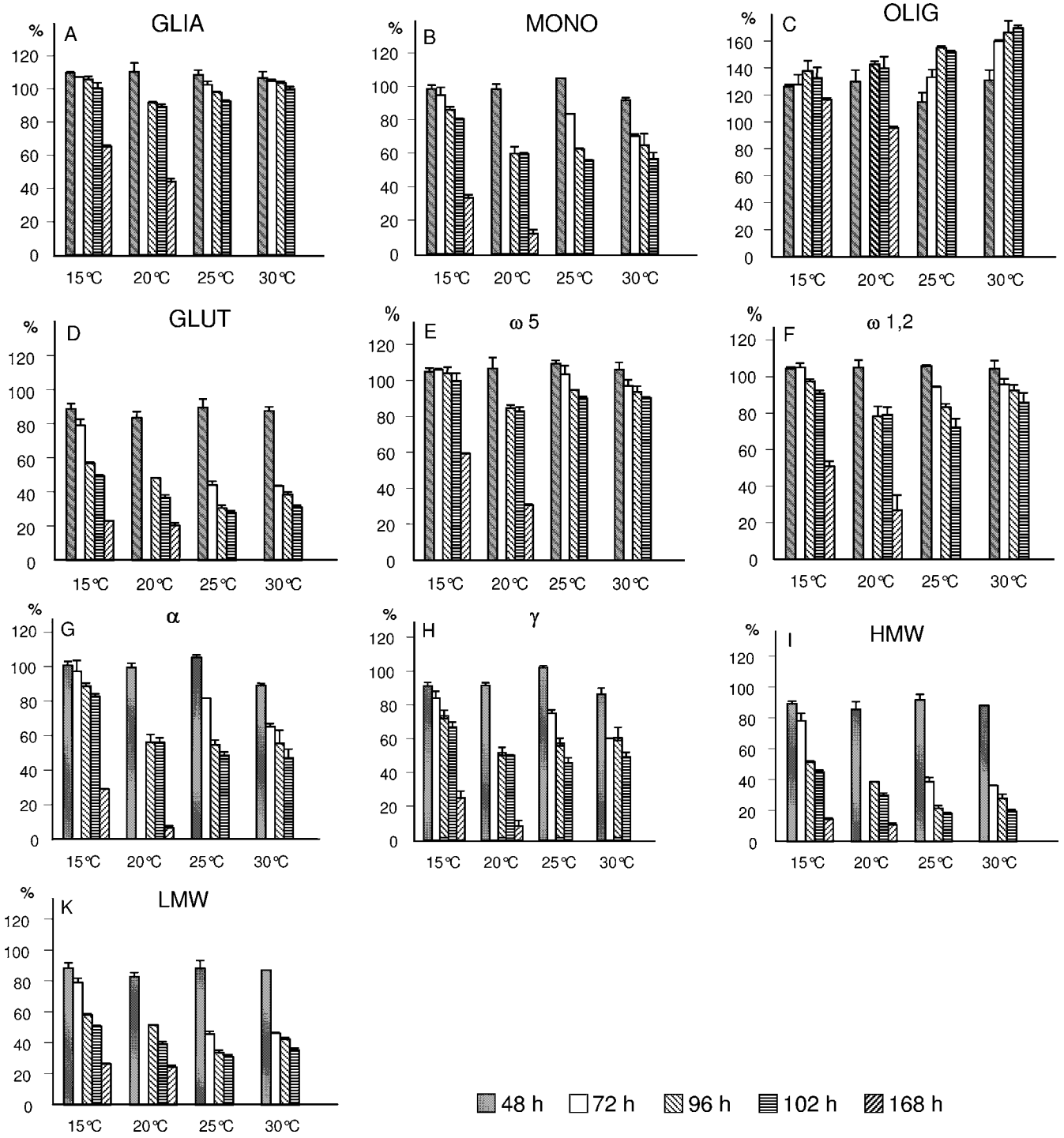

Figure 1. Degradation of total gliadins (**A**), monomeric gliadins (**B**), oligomeric gliadins (**C**) total glutenins (**D**), and different gluten protein types (**E**–**K**) of germinated wheat kernels as affected by the duration and temperature of germination (ungerminated samples = 100%; mean value of two determinations).

reliable folate data to be obtained. Furthermore, systematic quantitative studies on the degradation of different protein types during germination have not been carried out up to now.

The aim of a study carried out by our group was to increase the concentration of physiologically active constituents of wheat (folates and dietary fiber) by germination and to maintain acceptable technofunctional properties of the germinated material. The first part of the study presented here describes changes of the composition of wheat during germination. In particular, the effect of germination temperature and time should be studied in order to suggest conditions that are required to produce high-quality food.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained commercially from the sources given in parentheses: acetonitrile, formic acid, hydrochloric acid, $KHCO_3$, K_2HPO_4, 2-mercaptoethanol, methanol, $NaHCO_3$, NaH_2PO_4, sodium acetate, Na_2SO_4 (Merck, Darmstadt, Germany); α-amylase type II-A from *Bacillus* spp., 2-(cyclohexylamino)ethanesulfonic acid (CHES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), protease type XIV from *Streptomyces*, sodium ascorbate (Sigma, Deisenhofen, Germany); pteroyl triglutamate (Schircks, Switzerland). [$^2H_4$]-5-Methyltetrahydrofolic acid, [$^2H_4$]-5-formyltetrahydrofolic acid, [$^2H_4$]-10-formylfolic acid, [$^2H_4$]-tetrahydrofolic acid, and [$^2H_4$]-folic acid were synthesized as reported recently (*12*). Extraction buffer consisted of aqueous HEPES (50 mmol/L) and

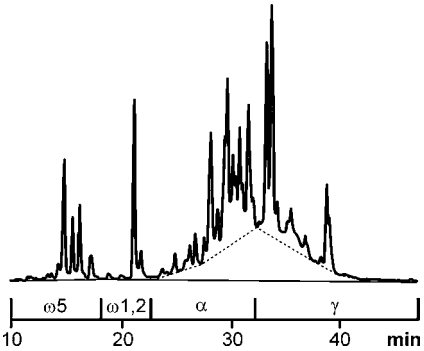

Figure 2. RP-HPLC of gliadins from germinated wheat (25 °C, 48 h). Continuous line, baseline for total gliadins; dashed line, baseline for monomeric gliadins; elution ranges of ω5, ω1,2, α, and γ-type gliadins are indicated.

aqueous CHES (50 mmol/L) at pH 7.85 and contained sodium ascorbate (2%) and 2-mercaptoethanol (20 mmol/L). Conditioning buffer for SAX cartridges was prepared by mixing aqueous solutions of NaH_2PO_4 (0.01 mol/L, 62 mL), K_2HPO_4 (0.01 mol/L, 28 mL), and 2-mercaptoethanol (0.2 mL), adjusting the mixture to pH 7.5 and finally making it up to 100 mL with water. Chicken pancreas solution was prepared by dissolving chicken pancreas (5 mg, DIFCO, Detroit, MI) in phosphate buffer (0.1 mol/L, pH 7.0) containing 1% ascorbic acid.

Germination of Wheat. Wheat kernels (cv. 'Tommi') from the 2004 harvest (150 g) were immersed with distilled water (450 mL) for 5 h at 20 °C and 70% relative humidity in a wide glass vessel ($\varnothing$ = 18 cm). Then the water was decanted, and the kernels were transferred into a stainless steel sieve (mesh size = 1.2 mm), washed with distilled water, and allowed to equilibrate for 19 h at 13 °C and 100% humidity. After washing with distilled water, the kernels were again soaked (4 h) and equilibrated (20 h) as described above. The wet kernels were finally soaked for 10 min, transferred into a plastic container with a perforated bottom (140 × 140 × 60 mm), and subjected to temperatures of 15, 20, 25, or 30 °C for a total time (soaking, equilibrating, plus germination) of up to 168 h. Aliquots for compositional analysis were taken after 48, 72, 96, 102, and 168 h. Germinating kernels were washed with distilled water twice a day. Germination was stopped by pouring liquid nitrogen onto the kernels, which were then immediately crushed in a blender (300 W, Krups, Solingen, Germany) and lyophilized. The dry material was milled into wholemeal flour by means of a laboratory mill (Quadrumat Junior, Brabender, Duisburg, Germany) and stored at −20 °C.

Protein Analysis. Wholemeal flours were extracted stepwise with 0.4 mol/L NaCl plus 0.067 mol/L $HKNaPO_4$, pH 7.6 (2 × 1.0 mL), at 20 °C (albumins, globulins), with 60% (v/v) ethanol (3 × 0.5 mL) at 20 °C (gliadins), and with 50% (v/v) 1-propanol containing Tris-HCl (0.05 mol/L, pH 7.5), urea (2 mol/L), and 1% (w/v) dithioerythritol (2 × 1.0 mL) at 60 °C under nitrogen (glutenin subunits) (*13*). After centrifugation, the corresponding supernatants were combined and diluted to 2.0 mL with the respective extraction solvents. Aliquots of the albumin/globulin (150 μL), gliadin (50 μL), and glutenin extract (100 μL) were then separated and quantified by RP-HPLC on C_8 silica gel at 50 °C using a solvent gradient of increasing acetonitrile concentration in the presence of 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.0 mL, and the detection wavelength was 210 nm. At least two determinations were performed for each protein fraction.

Extraction for Folate Quantitation. Lyophilized and ground sprouts (0.5−1.0 g) were overlaid with 10 mL of extraction buffer containing [$^2H_4$]-5-methyltetrahydrofolic acid, [$^2H_4$]-5-formyltetrahydrofolic acid, [$^2H_4$]-10-formylfolic acid, [$^2H_4$]tetrahydrofolic acid, and [$^2H_4$]folic acid (50−500 ng each).

Deconjugation of Pteroylpolyglutamates after Strong Anion Exchange. Sample suspensions were incubated with amylase (0.03 g) for 2 h at 37 °C and with bacterial protease (0.1 g) for 6 h at 37 °C. Subsequently, the samples were heated to 100 °C for 10 min, then adjusted to pH 7.0, and purified by solid-phase extraction using Bakerbond SAX cartridges (quaternary amine, 500 mg, no. 7091-3, Baker, Gross-Gerau, Germany) (*11*). The cartridges were successively activated with 2 volumes of hexane, methanol, and water and then conditioned with 7−8 volumes of conditioning buffer. After the sample extracts had been applied, the columns were washed with 6 volumes of conditioning buffer, and the folates were eluted with 2 mL of aqueous sodium chloride (5%, containing 1% sodium ascorbate, and 0.1 mol/L sodium acetate). After cleanup, 100 μL of 2-mercaptoethanol was added to each eluate, which was then adjusted to pH 7.0 by the addition of aqueous sodium hydroxide (0.1 mol/L) and spiked with rat serum (100 μL) and chicken pancreas solution (2 mL). The deconjugation was performed at 37 °C overnight. For testing conjugase activity, pteroyltriglutamate (1 μg) was added to food extracts prior to deconjugation. At the end of the conjugase treatment, the extracts were passed through a syringe filter (0.4 μm, Millipore, Bedford, MA). The purified extracts were then subjected to LC-MS/MS. Each food sample was analyzed in duplicate.

LC-MS/MS. The samples (10 μL) were chromatographed on a Nucleosil 100-5 C-18 reversed-phase column (250 × 3 mm; 5 μm, Macherey & Nagel, Düren, Germany) that was connected to a photodiode array detector and a TSQ quantum triple-quadrupole mass spectrometer (Thermo Electron, Dreieich, Germany). The mobile phase consisted of variable mixtures of aqueous formic acid (0.1%; eluent A) and acetonitrile (eluent B) at a flow of 0.4 mL/min. Gradient elution started at 9% B held for 4 min, followed by raising the concentration of B linearly to 25% within 10 min and to 100% within further 6 min. Subsequently, the mobile phase was held at 100% B for 4 min before the column was equilibrated for 5 min at the initial mixture. During the first 4.5 min of the gradient program, the column effluent was diverted to waste. The spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM) as reported previously (*14*). Spray voltage was set to 3200 V, sheath gas pressure was 60 mTorr, and auxiliary gas pressure was 20 mTorr. Capillary temperature was 350 °C and capillary offset 35 V. Source collision-induced dissociation (CID) was used with the collision energy set at 10 V.

RESULTS AND DISCUSSION

Germination of Wheat. Germination was performed in two phases. During the first one, which was the same in all experiments, the kernels were repeatedly soaked with water at 20 °C and stored at 13 °C to provide water for germination and to achieve a uniform distribution of the water in the kernels. In the second phase different temperatures of 15, 20, 25, or 30 °C were applied for different times. To prevent local drying of the seeds and also bacterial growth, they were washed with water twice a day and gently mixed. The water uptake during germination was monitored. At the end of the first phase and beyond, the water content of the kernels had increased from 15.2 to 51−58%. After 102 h, the kernels had increased in size by approximately 20%, and the embryos were well developed. Due to the relatively broad range of the water content and to increase the shelf life, the sprouts were lyophilized, milled into wholemeal flour prior to analysis, and stored at −20 °C. According to ref *15*, germinated material is stable under these conditions.

Protein Degradation. Wholemeal flours of germinated wheat were analyzed for the contents of albumins/globulins, gliadins, and glutenin subunits by a combined extraction/HPLC procedure (*13*). The amount of albumins/globulins increased slightly during germination; the variations due to different germination times and temperatures, however, were not significant ($p > 0.05$). In contrast, a substantial degradation of gluten proteins (gliadins, glutenins) could be observed during the 168 h germination. The degradation of total gliadins started after 102 h; a temperature of 20 °C was most effective (**Figure 1A**). Previous studies (*16*) revealed that the gliadin fraction consists not only of monomeric gliadins but also of so-called HMW-gliadins or ethanol-soluble

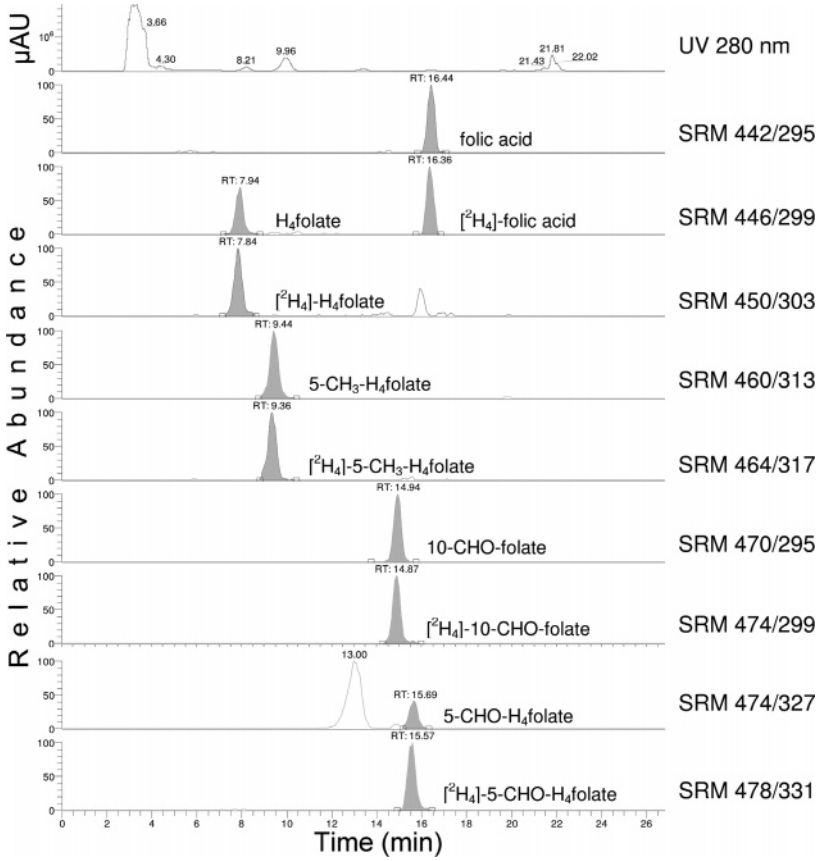

Figure 3. LC-MS/MS chromatogram of a germinated wheat extract. UV, UV absorption. Selected reaction monitoring (SRM) traces, *m*/*z* precursor ion/*m*/*z* product ion.

glutenins. These are oligomeric proteins including disulfide-linked α- and γ-gliadins and LMW subunits. These oligomers are eluted from the reversed-phase column together with monomeric α- and γ-gliadins. In the present study, the proportions of monomeric and oligomeric gliadins were roughly estimated by a specific integration of the HPLC chromatograms (**Figure 2**). The results indicated that monomeric gliadins were much more reduced by germination than total gliadins (**Figure 1B**). For example, only 12% could be detected after 168 h of germination at 20 °C. In contrast, oligomers increased during germination (exception: 20 °C, 168 h; **Figure 1C**), obviously due to a shift of degraded glutenins into the ethanol-soluble fraction. At 30 °C the degradation of monomers and the shift of degraded glutenins were balanced, so that the amount of total gliadins remained almost unchanged (**Figure 1A**). The optimal temperature for glutenin degradation, which started at 48 h (**Figure 1D**), was 25−30 °C. Single gluten protein types (monomeric ω-, α-, and γ-gliadins, HMW and LMW subunits of glutenin) were differently affected by germination depending on the temperature and time, but, in general, gliadin types were strongly degraded at 20 °C and glutenin types at 25 °C. ω5- and ω1,2-gliadins (**Figure 1E**,**F**) were less sensitive to proteolytic degradation than α- and γ-gliadins (**Figure 1G**,**H**), and LMW subunits were less sensitive than HMW subunits (**Figure 1I**,**K**).

Folate Content. Analysis of folates was performed by using a stable isotope dilution assay, which has recently been optimized for obtaining accurate data of monoglutamatic as well as of polyglutamic vitamers (*14*). This method has been applied to germinated wheat for the first time. Entire germinated seeds were used because it was not intended to use fractionated material in a subsequent study on the baking performance. A resulting LC-MS/MS chromatogram of sprouted wheat is shown in **Figure 3**. During germination a decisive increase in total folates was observed. In particular, when wheat was germinated at 20 °C, folate content exceeded 200 μg/100 g of dry mass after 102 h (**Figure 4**), which translated to a 3.6-fold increase compared to the initial content of 58 μg/100 g in ungerminated wheat. The folate content went through a maximum, and after 168 h, decreased again, but still was elevated as compared to the initial content of ungerminated wheat. Thus, a germination of 102 h at 20 °C resulted in a maximum of folates and, in this respect, to an increased nutritional value of wheat. The small amount of 100 g of dried sprouts would cover 50% of the daily 400 μg recommended intake of folates (*17*) and, therefore, could

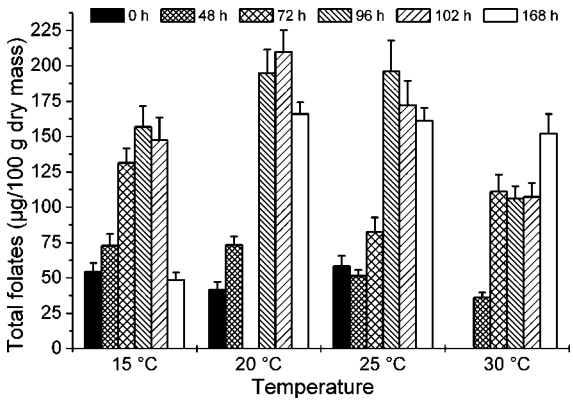

Figure 4. Total folates in wheat germinated for different times and at different temperatures. Stable isotope dilution assays were performed in duplicates.

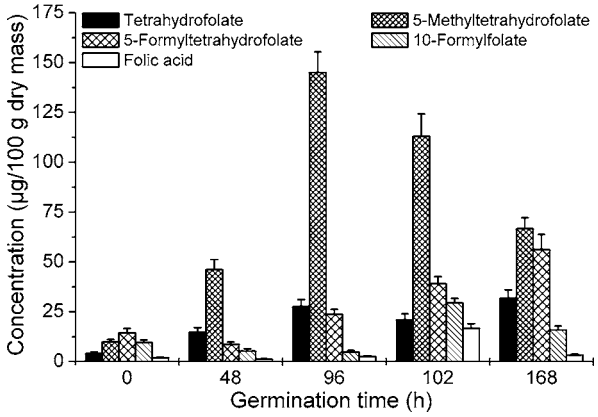

Figure 5. Folate distribution in wheat germinated for different times at 20 °C.

decisively improve the dietary supply of this important group of vitamins. This effect was similar at the temperatures of 15 and 25 °C with the difference that folates peaked at 96 h and the maximum contents were somewhat lower. In contrast to the latter temperatures, at 30 °C folates reached their maximum not before 168 h. These results are in good accordance with those of Mühlhäuser (*15*), who reported a folate content of 164 μg/100 g of dry mass in germinated wheat. Further good agreement was found with the studies of Liukkonen et al. (*8*), who investigated the folate content of rye during germination. Similarly to our results, the latter authors found the highest folate content after germination at 25 °C and a 3.4-fold increase at this temperature.

The use of SIDA enabled us further to measure the distribution of different folates, which has not been investigated before. Whereas in ungerminated wheat 5-formyltetrahydrofolate was the most abundant vitamer, the folate increase during germination mainly could be attributed to mounting 5-methyltetrahydrofolate, which declined again after 96 h of germination, whereas the other vitamers were still going up (**Figure 5**). This finding is well in line with previous reports that during the germination and growth of plants, 5-methyltetrahydrofolate is the predominant folate vitamer. Although tetrahydrofolate is the first vitamer in folate biosynthesis, 5-methyltetrahydrofolate is supposed to be most important to enable methionine biosynthesis and methylations via *S*-adenosyl methionine, both of which appear to be critical reactions in cell proliferation and cell growth. After germination, formylated folates appear to be important to enable light-dependent growth and purine biosynthesis.

Dietary Fiber. Previous studies reported a 25% increase of the dietary fiber content after 96 h of germination (*9*). The results of the present study are summarized in **Figure 6**. The concentration of the total dietary fiber decreased in the first 48 h of germination. This effect was more pronounced at low temperatures (15 and 20 °C) than at high temperatures (25 and 30 °C). Particularly at high temperatures a distinct increase of the total dietary fiber concentration was present after 102 h of germination. At 25 °C the concentration increased by >25% from 150 to 190 mg/g. At lower temperature the increase was lower. However, when the time of germination was increased to 192 h, the total dietary fiber contents at 20 and 30 °C were comparable to the concentration at 25 °C and 48 h.

The concentration of soluble dietary fiber did not increase during the first 96 h of germination. Beyond this point a substantial increase was observed. At 15 and 20 °C the concentrations increased by a factor of 3−4. In contrast to

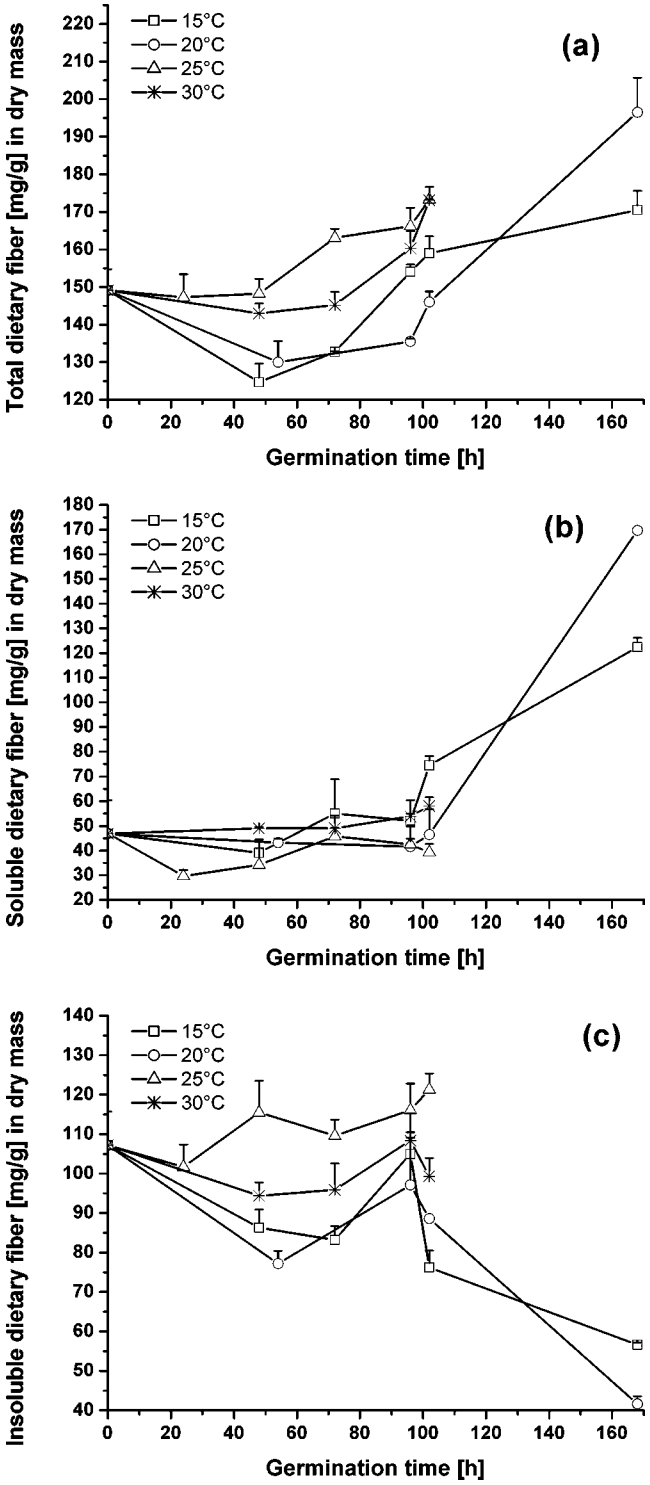

Figure 6. Concentration of dietary fiber (mg/g of dry mass) in wheat kernels (cv. 'Tommi') as affected by the duration and temperature of germination.

soluble dietary fiber, insoluble dietary fiber decreased, in particular when long germination times were applied. However, the extent of this decrease was lower than the increase of soluble dietary fiber, resulting in an overall increase of the amount of total dietary fiber. A maximum beneficial content of dietary fiber was obtained at longer germination times, which was contrary to the folate optimum. Therefore, from a nutritional point of view, an optimum folate and fiber content cannot be achieved simultaneously. It has to be concluded that the germination conditions have to be adjusted to the specific

nutritional purpose, whether the intake of folates or of fibers has to be increased. A sufficiently high intake of dietary fiber has significant health effects. Previous studies have indicated that increasing amounts of nutritional dietary fiber lower the risk of colon cancer (*1*) and coronary heart disease (*18*). In addition, a high level of dietary fiber delays the food-induced increase of glucose in the blood (*2*). This is beneficial for health, as a quick increase of blood glucose after meals has been shown to be associated with the insulin-dependent type II diabetes mellitus (*19*).

Concluding Remarks. Germination of wheat at 20 °C for 102 h leads to a substantial increase of the folate content but only to a slight increase of the concentration of dietary fiber. Proteins are differentially degraded depending on temperature and time of germination. To inhibit a strong increase of the gliadin/glutenin ratio, low germination temperatures (15−20 °C) can be recommended, which favor gliadin degradation and, therefore, might maintain acceptable technofunctional properties.

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