JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Oxygen Deficiency in Barley (*Hordeum vulgare*) Grain during Malting

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The steep water is generally aerated in industrial barley malting. However, it is questionable whether oxygen actually reaches the embryo, which remains entrapped under the husk, testa, and pericarp until chitting occurs. The aim of our study was to investigate whether barley embryos experience oxygen deficiency during steeping, and whether various steeping conditions affect the oxygen deficiency. Alcohol dehydrogenase Adh2 was induced in all steeping conditions studied. Therefore, oxygen deficiency occurred regardless of the steeping conditions. However, steeping conditions affected the rate of recovery from oxygen deficiency, germination rate, and onset of α-amylase production. When barley was subjected to oxygen deficiency by applying N_2 gas during steeping, the timing of the treatment determined its effects. The importance of aeration increased as the process proceeded. Oxygen deprivation at the beginning of the process had little effect on malt quality. Therefore, the timing of aeration is important in the optimization of germination during the steeping stage of malting.

KEYWORDS: ***Hordeum vulgare*; Poaceae; barley; germination; steeping; oxygen deficiency; alcohol dehydrogenase; ethanol detection**

INTRODUCTION

Seeds are generally considered to suffer from oxygen deficiency at the beginning of germination, before the growing embryo protrudes through the covering layers of the seed (*1*). This is known to apply also for germinating barley grain. Several findings indicate that the testa and pericarp limit the oxygen availability of the barley embryo and aleurone cells during the time between imbibition and completion of germination (*2*, *3*). The complex microbial community naturally present in cereal grains may also have an effect on oxygen availability (*4*, *5*). Moreover, oxygen availability may play an important role in seed coat imposed dormancy, which is the prevailing form of dormancy in cereals (*6*).

Barley aleurone cells react to oxygen deficiency by expressing certain lactate and alcohol dehydrogenase isoenzymes (*7*, *8*), and ethanol has been detected in the prevailing gaseous atmosphere even in well-aerated laboratory germination conditions (*2*). It has been assumed that the ethanol originates from the barley grain. However, yeasts that naturally colonize the grain may also contribute to ethanol production. Although the barley plant does not cope well with anoxia, it has been demonstrated that barley kernels can survive for days without oxygen (*9*). This survival is largely dependent on the existence of the constitutive alcohol dehydrogenase (Adh; alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) Adh1, which is found in the scutellum, embryo, and endosperm of a dry barley seed. Adh reduces acetaldehyde to ethanol while oxidizing $NADH^+$ to NAD. When barley is subjected to anoxia, two other Adh isoenzymes are induced: Adh2 and Adh3 (*10*, *11*). In embryos and aleurone cells this induction occurs even under complete anaerobiosis, whereas complete anoxia kills root tissue. The embryo and aleurone cells of barley grains are therefore very well equipped to survive severe oxygen deficiency. However, as opposed to rice, the barley seed will not complete germination without oxygen (*12*, *13*). This may be a consequence of aleurone cells not being able to respond to gibberellic acid in the absence of oxygen (*7*). As a consequence, gibberellic acid induced changes such as de novo production of the starch-hydrolyzing enzymes α-amylase, limit dextrinase, and α-glucosidase as well as protease-mediated release of bound β-amylase to the free form do not take place under anoxia (*14*). This leads possibly to sugar starvation of the embryo, which eventually dies.

During aerobic germination, Adh activity and ethanol production decrease during the first 3−4 days after imbibition (*15*−*17*). This is a consequence of the down-regulation of the *adh1* gene by gibberellic acid (*16*). It is assumed that Adh1 accounts

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10.1021/jf0521505 CCC: $33.50
Published on Web 12/17/2005

for the activity during aerobic germination, since the inducible Adh2 and Adh3 enzymes have not been detected. These experiments have, however, in general been conducted in perlite or in moist sand, i.e., in conditions which differ significantly from those prevailing in the malting process. In industrial malting, barley is steeped with alternating immersion and air rest periods. Air rests were originally introduced into steeping to improve the oxygen availability. For the same reason, the steep water is often aerated. However, providing oxygen to the steeping water may be inefficient, because the solubility of oxygen in water is poor. It is questionable whether the air introduced into a steep actually reaches the embryo, which remains entrapped under the husk, testa, and pericarp until chitting has occurred. In addition, the aerobic microbial populations normally colonizing the barley kernel may compete with the grain tissues for dissolved oxygen during the steeping process (*4*, *5*). Steep water aeration has in some studies been shown to promote germination (*18*, *19*). However, these studies have applied very long immersion periods (20−60 h in ref *18*) or left the samples unaerated for long periods of time, which means that the effects of anoxia may be confused with the effects of CO_2. It has also been claimed that aeration during steeping in fact has minor effects on malt quality (*18*−*20*).

While the principles of aerobic steeping were discovered long ago, the effects of different malting procedures on barley grain physiology have not been investigated in depth. The aim of our present study was therefore to investigate the effect of oxygen deficiency on barley germination and malt quality by applying nitrogen gas at various steeping steps. In particular, the aim was to investigate whether the barley embryo reacts to oxygen deficiency during steeping by producing Adh2 and Adh3 isoenzymes, or whether the ethanol detected during malting is merely a product of Adh1 isoenzymes already present in the dry embryo. Moreover, the aim was to study how the timing of oxygen deficiency affected germination and malt quality.

MATERIALS AND METHODS

Barley Analyses. The germination capacity and germinative energy were determined with EBC methods 3.5.2 and 3.6.2 (*21*). The moisture content was determined by heating at 131 °C according to *Analytica EBC* (*21*) method 3.2. The protein content was determined with Kjeldahl method 3.3.1 (*21*). Before malting, barley samples were sieved to remove grains <2.5 mm.

Effect of the Steeping Conditions on Barley Embryo Oxygen Deficiency. Nondormant barley (*Hordeum vulgare* L, Poaceae) of the cultivar Scarlett (crop 2004, lot 452) was used for the study. Scarlett is one of the main malting barley varieties used currently in Europe. Samples (300 g) were malted in specially designed, computer-controlled micro-malting equipment with a separate drum for each sample (Hulo Engineering, Helsinki, Finland). Each drum had its own steeping water and controllable gas atmosphere. All barley samples were steeped at 18 °C for 8 h, followed by a 16 h air rest (20 °C) and a second steep (2 h, 18 °C). The barley was then allowed to germinate for 5 days at 16 °C and dried (kilned) in warm air (4 h, 50 °C; 3 h ramp to 60 °C; 2.5 h, 60 °C; 3 h ramp to 85 °C; 1 h, 85°C) in a separate kiln. The rootlets were removed before analyses.

To subject the barley to oxygen deficiency, nitrogen (N_2) gas (0.17 L/min per drum) was applied during the first 8 h steep ("N_2 first steep"), during the first 8 h of the air rest ("N_2 beginning of air rest"), or during the last 8 h of the air rest ("N_2 end of air rest"). To mimic unevenly distributed aeration in industrial malting conditions, one drum was left unaerated during the air rest to allow the concentration of carbon dioxide in the malting drum to rise ("no aeration in air rest"). One drum was treated with 0.5 g/L hydrogen peroxide (H_2O_2) in the first steep (with aeration) to apply oxidative stress ("H_2O_2"). The different treatments were compared to a continuously aerated control ("control"). The drums were aerated at a rate of 0.17 L/min during steeping and air rest and at 0.27 L/min during germination, unless a special treatment was applied. The malting experiment was performed in duplicate (experiments 1 and 2).

A Fourier transform infrared spectroscopy (FTIR) multicomponent gas analyzer, Gasmet (Temet Instruments Ltd., Helsinki, Finland), with a heated, flow-through, 5 m path length sample cell was used to monitor the ethanol and carbon dioxide concentrations in the headspace of each malting drum during steeping and germination. The percentage of germinated grains was calculated from a sample of 150−200 kernels daily, until the percentage of germinated grains reached 90%. Samples were taken during malting for Adh activity assay, separation of isoezyme dimers by gel electrophoresis, and α-amylase activity analysis.

Origin of the Ethanol Produced during Malting. To verify that barley (and not the microbes colonizing the grain) was responsible for most of the fermentative activity during malting, kernels of two barley samples of the Finnish malting barley cultivar Saana (crop 2001) were cut in half, and the embryoless (125 g) and embryo (112−125 g) halves were processed separately. The samples were steeped for 8 h at 16 °C, followed by a 16 h air rest, a second steep of 8 h, and 2 days of germination. Ethanol production was monitored continuously by FTIR as described above.

Monitoring Industrial-Scale Malting Conditions. To compare laboratory and industrial malting conditions, the headspace of four industrial malting batches was monitored with FTIR as described above. The raw material in all batches was Saana barley of crop 2001. The ethanol and carbon dioxide concentrations were monitored continuously at five locations within the grain bed during the air rest of steeping and at four locations above the grain bed during germination.

Electrophoretic Separation of Adh Isoenzyme Dimers. The different dimers formed by the three isoenzymes (Adh1, Adh2, and Adh3) were separated on the basis of differences in charge using native gel electrophoresis. Ten embryos per sample were homogenized in 500 μL of prechilled buffer (50 mM Tris−HCl, pH 6.8, 12%, v/v, glycerol) and centrifuged for 15 min at 10000*g*. Gel electrophoresis was performed as described by Harberd and Edwards (*10*), and activity staining was done according to ref *9*. Separation of Adh isomers was performed with samples taken during malting as well as with embryos from dry, native barley. Embryos were separated from dry barley by cutting after removal of the husk. Therefore, embryos from native barley samples were contaminated with endosperm and aleurone cells. Several barley samples representing different varieties were analyzed to verify the general applicability of the results. The samples included nondormant Scarlett (lot 452, crop 2004) and dormant Scarlett (lot 447, crop 2004) as well as samples of the malting barley cultivars Saana (crop 2001), Annabell (crop 2004), and Inari (crop 2001). An anaerobic control was prepared by germinating Saana 2001 barley for ∼24 h and then sparging it with N_2 gas for ∼24 h. Gels were scanned using a BioRad GS-710 calibrated imaging densitometer (San Francisco), and images were collected using the Quantity One program, version 4.1.0. (BioRad).

Adh Activity. A method described by Crawford (*22*) was modified for barley embryos. Ten embryos were excised from frozen process samples and collected into a test tube containing 1 mL of cold buffer (0.1 M Tris−HCl, pH 8.0, 2 mM dithiothreitol, 1.5% polyvinylpolypyrrolidone) and some glass sand (made from crushed glass pipets). The embryos were ground using a plastic pestle, and the cell debris was separated by centrifuging (20000*g*, 15 min). The extract was diluted 1:50 in buffer and used for Adh activity assessment at 30 °C. Diluted extract (0.1 mL), 1.0 mL of buffer (0.1 M Tris−HCl, pH 8.0, 2 mM DTT), and 0.2 mL of NADH (1.2 mM) were mixed in a cuvette, and the absorbance was recorded for 3 min at 340 nm to record the sample background. After 3 min 0.2 mL (100 mM) of acetaldehyde substrate was added, and the absorbance was recorded for 10 min. The Adh activity was expressed as nanokatals per embryo. The possible interference of microbial Adh activity was studied by comparing the Adh activity of husks from 20 germinating barley kernels to that of 10 embryos from the same sample. Before analysis, the barley sample was steeped in water for 5 h at 14 °C to activate both the embryo and the microbes present in the husks.

α-Amylase Activity. Samples from the malting process were freeze-dried and ground in a disk mill (Bühler Miag, Braunschweig, Germany)

Table 1. Malting Quality of the Barley Samples Used in the Study

barley	crop	moisture content (%)	protein content (% dw)	GC[a] (%)	GE(4 mL)[b] (%)
Scarlett, lot 452	2004	12.9	10.9	99	97
Scarlett, lot 447	2004	11.5	10.1	97	96
Saana	2001	12.5	10.5	nd[c]	nd[c]
Annabell	2004	13.5	9.1	nd[c]	nd[c]
Inari	2001	15.0	11.9	nd[c]	nd[c]

[a] Germination capacity (hydrogen peroxide method). [b] Germination energy determined using 4 mL of water for 100 grains. [c] Not determined.

using a 0.2 mm gap between the disks. The α-Amylase activity was analyzed with a Ceralpha kit (Megazyme Co., Wicklow, Ireland) using an extraction time of 30 min and assay conditions as specified by the manufacturer.

Malt Analyses. Malt samples were analyzed using the following EBC-recommended methods: fine/coarse extract content, friability, and soluble nitrogen and free amino nitrogen contents (*21*).

RESULTS

Effect of the Steeping Regime on Germination and α-Amylase Activity. Scarlett barley (lot 452) was malted using a range of steeping conditions with the aim of understanding the effect of the steeping conditions on the degree of oxygen deficiency in barley embryos. The grain was considered to have good malting quality with a protein content of 10.8% (**Table 1**). On the basis of the high germinative energy (97%), the barley lot was not dormant. The different treatments applied during steeping affected grain germination. **Figure 1** shows the average percentages of germinated grains from the two laboratory-scale malting experiments. H_2O_2 in the first steep water speeded up germination considerably, although the barley was not dormant. Sparging with N_2 gas did not impair germination when applied during the first steep only. However, when N_2 gas was applied at the end of the air rest, it slowed germination, which happened, too, when aeration was omitted during the air rest. The germination rates were reflected in the α-amylase activities measured after 3 days of malting (steeping + germination) (**Table 2**). The barley that was steeped in H_2O_2 had the highest α-amylase activity (139−155 U/g dw), and the barley that was either not aerated or sparged with N_2 gas during the air rest

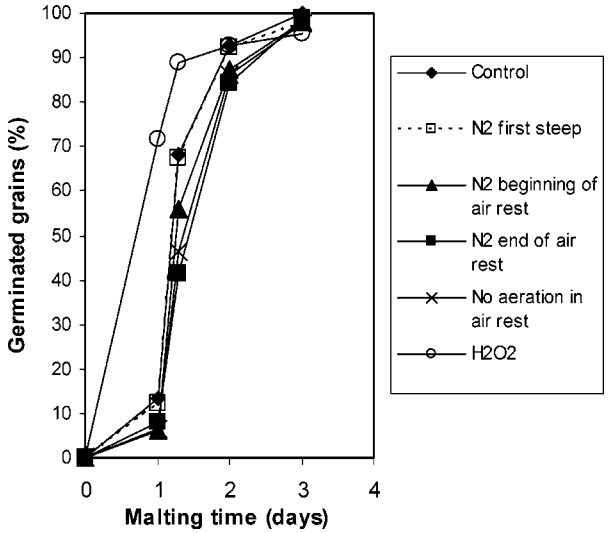

Figure 1. Percentage of germinated grains during laboratory-scale steeping and germination. The results are average values of malting experiments 1 and 2.

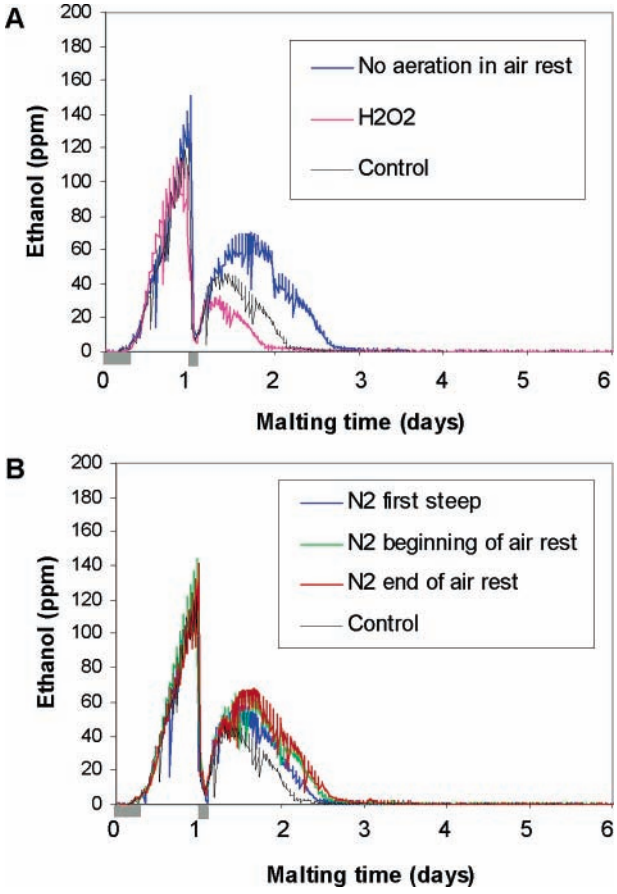

Figure 2. Ethanol concentration (ppm) in the headspace of malting drums during laboratory-scale steeping and germination. Steeps are indicated as gray bars below the *x* axis. The results represent one of the duplicate malting experiments.

Table 2. α-Amylase Activity (U/g dw) of Germinated Barley (3 days) and Kilned Malt

	3 day barley[a,b]		kilned malt[a,c]	
	experiment 1	experiment 2	experiment 1	experiment 2
control	102 ± 6	114 ± 4	324 ± 11	321 ± 8
N_2 first steep	125 ± 4	98 ± 2	311 ± 14	308 ± 7
N_2 beginning of air rest	83 ± 2	76 ± 2	335 ± 8	312 ± 19
N_2 end of air rest	88 ± 4	68 ± 1	319 ± 28	319 ± 9
no aeration in air rest	83 ± 4	56 ± 2	332 ± 17	309 ± 4
H_2O_2	155 ± 6	139 ± 5	328 ± 8	293 ± 2

[a] Each sample was analyzed in triplicate. The values in the table are means of two malting experiments ± standard deviation. [b] Barley malted for 3 days and lyophilized. [c] Barley malted for 6 days and dried with hot air (kilned).

had the lowest activities (56−88 U/g dw). At the end of malting, there were no significant differences in α-amylase activities between the samples. The average α-amylase activity before kilning was 396 U/g dw (data not shown). Kilning reduced the α-amylase activity by 19% on average.

Production of Volatile Metabolites during Malting. The gas atmosphere prevailing in each malting drum was monitored continuously during the steeping and germination phases. The main volatile produced during malting was carbon dioxide (CO_2), the concentration of which increased from the beginning of the first steep and reached a level between 0.1% and 0.3% by the end of the air rest in all aerated or N_2-sparged samples (data not shown). In the malting drum that was not aerated during the air rest, the CO_2 concentration reached 1%. The CO_2 concentrations dropped when the second steep water was

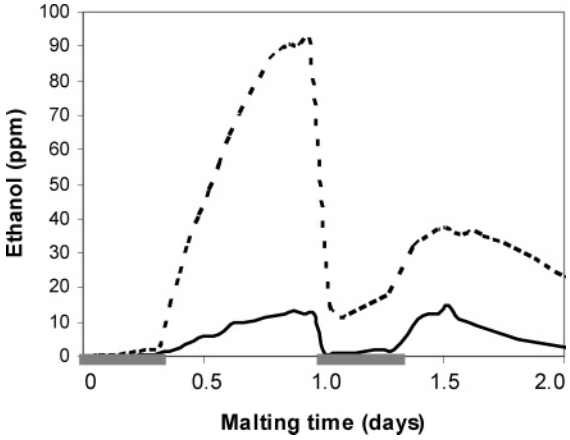

Figure 3. Ethanol concentration (ppm) in the malting drums during laboratory-scale steeping and germination of embryoless half-kernels (solid line) and half-kernels with embryos (dotted line). Steeps are indicated as gray bars below the *x* axis. The results represent one of the duplicate malting experiments.

introduced and began to rise again during germination. The highest CO_2 concentrations (0.4−0.7%) were reached after 3−4 days of malting, after which the concentrations decreased to 0.2−0.3%. With the exception of omitting aeration, the steeping conditions had no significant effect on CO_2 production.

The second most abundant volatile compound detected was ethanol (**Figure 2**). The highest ethanol concentration (120−140 ppm) was reached at the end of the air rest. It dropped at the introduction of the second steeping water and started to rise again after the steep. A second concentration peak occurred during the second day of malting. All samples released ethanol at similar rates during the air rest. However, there were differences in the ethanol disappearance rates between the samples during germination. The ethanol concentration decreased most rapidly in the sample that had been steeped in H_2O_2 (**Figure 2A**). The slowest ethanol concentration decrease was noted in the samples that had been sparged with N_2 gas during the air rest (**Figure 2B**) and the sample that was not aerated during the air rest (**Figure 2A**). The results in **Figure 2** represent one of the duplicate malting experiments. The results of the other malting experiment were similar (data not shown).

Origin of the Ethanol Detected during Malting. The ethanol production of embryoless half-kernels was compared to that of half-kernels with embryos to verify the role of the embryo, and on the other hand aleurone cells and microbes as producers of ethanol. Our study showed that most of the ethanol detected during malting originated from the embryo. Emryoless half-grains produced ∼13% and 25% of the detected ethanol during steeping and germination, respectively (**Figure 3**). We estimated that aleurone cells and the microbial community may account for up to 25% of the ethanol produced during germination.

Monitoring Industrial-Scale Malting Conditions. In industrial steeping conditions, the CO_2 concentration reached on average 3% at the end of the air rest (**Figure 4**). Therefore, the carbon dioxide concentration was higher in industrial-scale malting than in the laboratory-scale experiments. The ethanol concentrations and ethanol production patterns in the industrial-scale malting processes were similar to those in the laboratory-scale experiment (**Figure 4**). The lines in **Figure 4** are average values of four to five locations within the steeping and germination vessels of four malting batches. The local ethanol concentration peaks varied considerably: concentrations between 15 and 200 ppm were measured during the air rest and between 15 and 100 ppm during the first day of germination (data not shown).

Effect of the Steeping Conditions on the Adh Activity. The Scarlett barley lot 452 that was used for laboratory-scale malting experiments 1 and 2 had an Adh activity of 24 ± 0.2 nkat/embryo in the dry grain. This Adh activity can be considered as the endogenous barley Adh activity, because very little Adh activity was detected in the husks of steeped barley grains. The microbial Adh activity of about 10 husks was only 0.7−2.4% of the Adh activity of 10 embryos (data not shown). Taking into account that healthy embryos are likely to contain significantly fewer microbes than husks, it can be concluded that the microbial contribution to the measured Adh activity is negligible.

The Adh activity increased in all samples during the first day (8 h steep + 16 h air rest) of malting (**Figure 5**). During the second day, the Adh activity decreased in all samples except the one not aerated during the air rest. The activity continued to decrease during the third day. At the same time the standard deviations increased, probably as a consequence of uneven germination.

Native polyacrylamide gel electrophoresis was used to detect the different Adh isozymes during malting (**Figure 6**). Native Scarlett barley contained large amounts of Adh1·Adh1 isomer but also some Adh1·Adh2 dimers as well as traces of Adh1·Adh3. Similar results were obtained for the other native barley samples (**Figure 7**). One Scarlett lot (447) also appeared to contain traces of Adh2·Adh2 or possibly Adh2·Adh3 dimer. The anaerobically treated control barley contained all isomers as expected. Both samples had also a faint band just below the thick Adh1·Adh1 homodimer. This band has previously been recognized as a conformer of the Adh1·Adh1 isomer (*11*). Barley that had been steeped for 8 h with various special

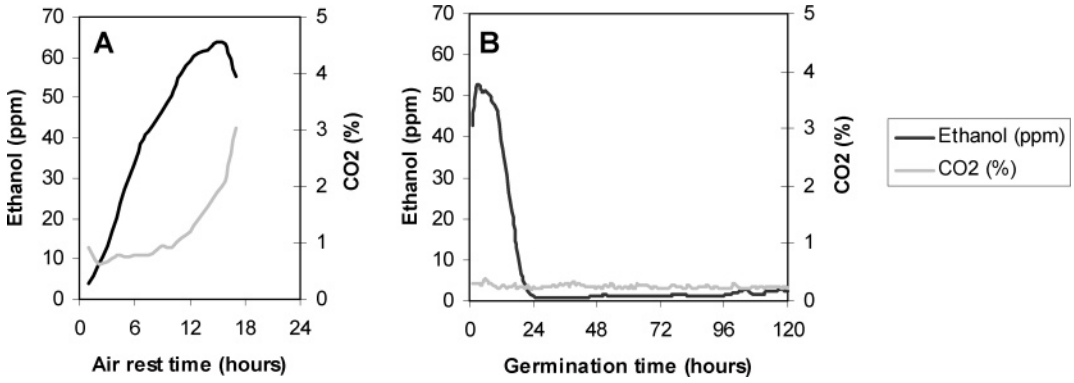

Figure 4. Ethanol and carbon dioxide concentrations in the headspace of industrial-scale malting drums during the steeping air rest (**A**) and germination (**B**). Each line represents the average value of four batches.

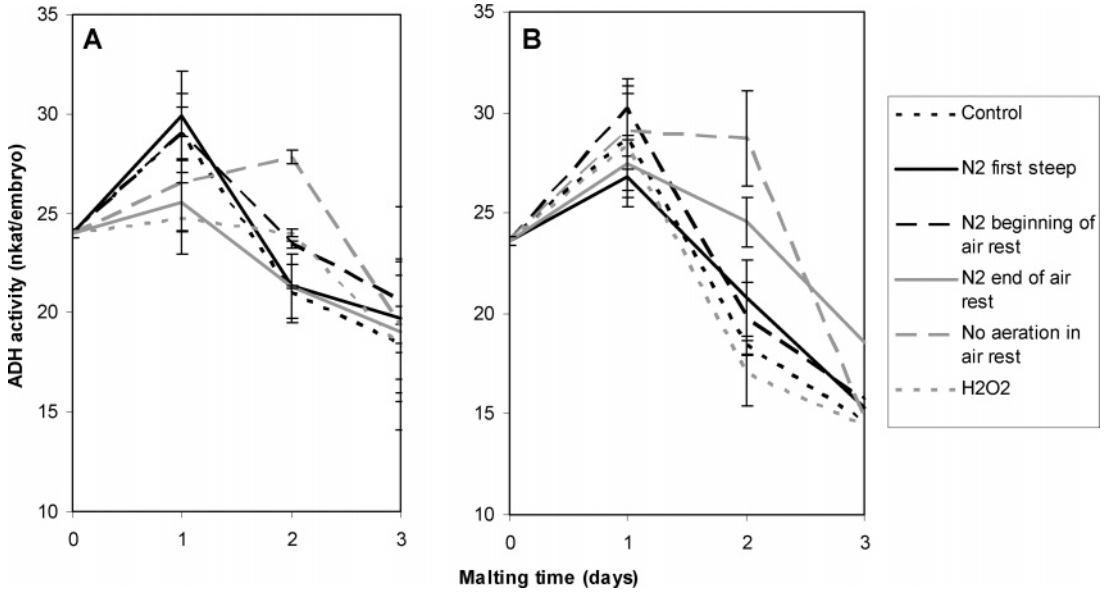

Figure 5. Adh activity of barley embryos during laboratory-scale malting. Standard deviations are indicated by error bars. Key: (**A**) malting experiment 1, (**B**) malting experiment 2.

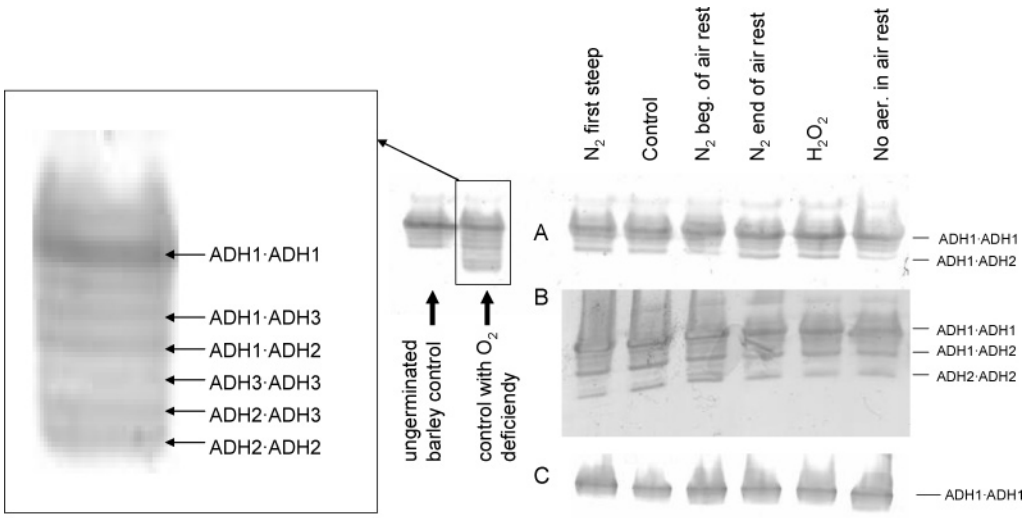

Figure 6. Detection of Adh isoenzymes during laboratory-scale malting: (left, enlarged) control barley sample with O_2 deficiency showing all Adh isozymes; (middle) ungerminated barley control showing only Adh1·Adh1 and Adh1·Adh2 isozymes and control sample with O_2 deficiency showing all Adh isozymes; (right) samples taken after eight (row A), 24 (row B), and 72 (row C) h of malting in various conditions.

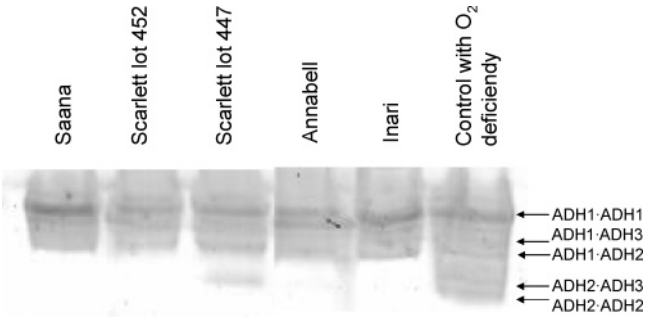

Figure 7. Detection of Adh isoenzymes in various ungerminated barley samples. The gel has been overstained to detect even small amounts of enzyme present.

Table 3. Malt Quality Analyses

	extract content[a] (% dw)	free amino N content[a] (mg/L)	soluble N content[a] (mg/L)	color[a] (EBC)	friability[a] (%)
control	82.5	180	866	2.8	87.7
N_2 first steep	82.4	174	854	2.7	87.4
N_2 beginning of air rest	82.5	179	869	2.8	87.1
N_2 end of air rest	82.7	182	869	2.8	85.9
no aeration in air rest	82.7	181	886	3.0	86.6
H_2O_2	82.3	180	862	2.6	88.3
std dev of std malt	0.30	5.2	10.5	0.21	0.94

[a] The values are averages of two malting trials.

treatments showed the same bands as the native barley (**Figure 6**, row A). After the 16 h air rest, a strong band representing the Adh2·Adh2 dimer had appeared in all samples (**Figure 6**, row B). At this point, the control as well as the samples that had been sparged with N_2 during the first steep and the beginning of the air rest showed bands representing all isomers. The bands containing the Adh3 isomer were very faint, indicating low levels of this isoenzyme compared to the Adh1 and Adh2 isoenzymes. As germination proceeded, the bands representing the Adh2·Adh2, Adh2·Adh3, and Adh3·Adh3 dimers gradually disappeared (**Figure 6**, row C).

Effect of the Steeping Conditions on Malt Quality. The quality of the malt produced in laboratory-scale malting experiments 1 and 2 was evaluated using standard malt analyses. The effects of the steeping conditions on the friability, extract, free amino nitrogen, and soluble nitrogen contents, and color of the malts were small (**Table 3**). The initial germination rate appeared to correlate with malt friability, the H_2O_2-treated sample showing the highest friability. The sample sparged with N_2 at the end of the air rest had the lowest friability.

DISCUSSION

The aim of our present study was to investigate whether the barley embryo reacts to oxygen deficiency during steeping by producing Adh2 and Adh3 isoenzymes, or whether the ethanol detected during malting is merely a product of Adh1 isoenzymes already present in the dry embryo. Native, ungerminated barley seeds, embryos, and aleurone cells have previously been found to contain mainly Adh1·Adh1 homodimer and traces of Adh1·Adh2 (*8*, *11*, *15*). We analyzed five barley samples representing different malting barley varieties, and found small amounts of Adh1·Adh2 and traces of Adh1·Adh3 as well as the dominating Adh1·Adh1 homodimer in the ungerminated kernels. This suggests that some of the Adh2 and Adh3 isoenzymes that are expressed during grain development in the starchy endosperm and pericarp/testa/aleurone (*23*) may survive grain desiccation into the mature kernel. However, both the thickness of the Adh1·Adh1 band compared to that of the other two and the absence of the Adh2 and Adh3 homodimers indicate that Adh1 is much more abundant than the other isoenzymes.

During the first steep, the quiescent grain became rehydrated, and metabolic activity was resumed. Low concentrations of ethanol were detected in the headspaces of the malting drums, indicating a fermentative metabolism. It was estimated that at least 75% of the ethanol production was due to the embryo rather than the aleurone cells or the microbial community of the grain. The Adh isoenzyme pattern of the embryo after 8 h of steeping still resembled that of the native barley. This applied also for the barley that had been sparged with N_2 gas during steeping or steeped in a solution of H_2O_2. During the 16 h air rest, all barley samples showed clear signs of a fermentative metabolism: the ethanol content in the malting drum headspace increased rapidly, the Adh activity rose, and a clear band representing the Adh2 homodimer appeared. Moreover, the Adh2·Adh3 and Adh3·Adh3 dimers were detectable at least in the samples sparged with nitrogen during the first wet steep at the beginning of the air rest as well as in the control. The Adh2 and Adh3 isoenzymes are both induced by oxygen deficiency (*11*, *15*), indicating that the barley embryos were experiencing oxygen deficiency during the steep or air rest, in all of the conditions studied. Hanson et al. (*8*) found that Adh2 and Adh3 enzymes were expressed in isolated aleurone layers at a headspace oxygen concentration of 5% or lower. Interestingly, Crabb and Kirsop (*3*) estimated that when the oxygen concentration of the aeration air was 4%, dehusked barley grains had oxygen uptake and carbon dioxide production similar to those of husked barley at an oxygen concentration of 21%. This suggests that the oxygen concentration in the immediate vicinity of the embryo of steeped barley is on the order 4−5%, a level which in aleurone cells induces the Adh2 and Adh3 isoenzymes.

Previously, only the Adh1 isoenzyme has been detected in barley grain during germination and early seedling development (*15*). This implies that the malting conditions may be more anaerobic than the germination conditions used in other studies. Alternatively, the Adh2 and Adh3 isoenzymes have not been previously detected due to relatively wide sampling intervals. The fact that even the continuously aerated control expressed all isoenzymes suggests that aeration cannot overcome the oxygen deficiency. It has indeed been suggested that aeration during steeping provides oxygen mainly for the microbial community present in the covering layers of the barley grain (*20*). The appearance of the Adh2 and Adh3 isoenzymes coincided with a rise in Adh activity. Previously, the Adh activity has been reported to decrease steadily during germination (*16*, *20*). Again, this supports the view that the malting conditions are more anaerobic than the germination conditions on perlite or filter paper.

After the air rest, 70% of the grains in the H_2O_2-treated sample had germinated, while the other samples had germination percentages between 6% and 13%. H_2O_2 is known to induce germination in dormant barley, a fact that is exploited in analytical methods such as germination capacity method 3.5.2 (*21*). H_2O_2 is an active oxygen source that can diffuse into plant tissue and affect germination directly or indirectly through O_2 production. Recent work on *Zinnia elegans* seeds indicates that H_2O_2 itself promotes germination rather than O_2 (*24*). H_2O_2 may be involved in the degradation of abscisic acid (*25*) or in the oxidation of inhibitors in the pericarp (*24*). It is also possible that the effect of H_2O_2 is related to its antimicrobial effects.

After the first 26 h of malting (8 h steep, 16 h air rest, 2 h steep), the aeration rate was doubled and the temperature lowered slightly, to mimic industrial germination conditions. During the second day of malting, the germination percentages of all samples rose sharply to a level of 84−93%. The ethanol content of the headspace rose to 30−70 ppm and then declined. The ethanol concentration in barley has been shown previously to follow a similar pattern; i.e., the grain ethanol content decreased during the first 1−2 days of germination (*17*). Moreover, ethanol production in industrial malting also followed the same pattern, indicating that our results are applicable to industrial practice. The order in which the ethanol concentration declined was consistent with the germination percentage at 1.3 days of malting. The decline in ethanol production is therefore probably a consequence of the down-regulation of the constitutive *adh1* gene by gibberellic acid, as observed previously in aleurone cells (*16*). Moreover, chitting of the grains, that is, rupture of the testa and pericarp and emergence of the root and shoot, may lead to better oxygen conditions for the embryo followed by down-regulation of the *adh2* and *adh3* genes. A high germination rate also correlated well with an early rise of α-amylase activity, an enzyme which is up-regulated by gibberellic acid (*26*).

During the second day of malting, the Adh activity declined also in all samples except in the one not aerated during the air rest. The absence of aeration or sparging with N_2 gas led to a buildup of carbon dioxide at a final concentration of 1% in the headspace of the malting drum. The barley sample that had not been aerated during the air rest had a germination rate and onset of α-amylase production comparable to those of the samples that had been sparged with N_2 gas even though the oxygen concentration cannot have decreased considerably during the rest. Ekström et al. (*27*) measured a maximum oxygen consumption of ∼2.5 (mmol/kg)/h in steeped barley (3 days of steeping, 12−14 °C, continuous oxygen supply). If the barley in the present experiments had a similar consumption rate, the oxygen concentration in the malting drum headspace would still be above 19% after 16 h. It therefore seems more likely that the carbon dioxide concentration, which rose to 1% during the air rest, was responsible for the delayed germination. CO_2 has

indeed previously been shown to impair germination and modification (*28*), and extraction of CO_2 during the air rest has been found to improve the malt extract content on the industrial production scale (*29*). Another possibility is that the absence of aeration left a thicker water film on the grains during the air rest, leading to decreased oxygen uptake rates.

Our results show that the timing of externally applied oxygen deficiency determines its effects. While oxygen deficiency during the first steep appeared to have no effect at all on germination, oxygen deficiency at the end of the air rest clearly slowed germination. In these experiments, oxygen deficiency was not applied later than the end of the air rest. If oxygen deficiency would occur later in the process, it would probably affect germination and modification significantly, since less Adh activity would be available to maintain a fermentative metabolism. Therefore, the importance of aeration increases as the process proceeds. Anaerobic pockets have been detected in industrial-scale aerated steeping vessels (*30*). During air rest periods, air is sucked through the steeping vessel from the top to the bottom, and consequently, the grain at the bottom of the vessel may become starved of oxygen (*17*). The germination process is, however, generally considered to be more aerobic.

While the different steeping regimes affected the germination rate and rate of recovery from oxygen deficiency, they had little effect on the quality of the final malt. Oxygen deficiency applied at the end of the air rest had a slight decreasing effect on malt friability, whereas oxygen deficiency earlier in the process had a negligible effect on malt quality. Several other workers have reported minor effects of anaerobic treatment during steeping (*18*−*20*). Gibbons (*28*) also reported relatively small effects of nitrogen gas application during steeping.

In conclusion, we have shown that barley grain suffered from oxygen deficiency during the steeping process despite continuous aeration. It is therefore likely that the air introduced into the steep does not reach the embryo. The absence of oxygen during the first steep had no effect on germination or malt quality, whereas oxygen deficiency at the end of the air rest retarded germination. Therefore, the importance of aeration increased as the process proceeded. Aeration also plays an important role in removing CO_2 that has a negative effect on germination and modification. This is of particular importance in industrial-scale malting, where high CO_2 concentrations may easily develop, e.g., at the end of the steeping period.

ABBREVIATIONS USED

Adh, alcohol dehydrogenase enzyme; *adh*, alcohol dehydrogenase gene; FTIR, Fourier transform infrared spectroscopy.

ACKNOWLEDGMENT

We thank Tarja Wikström, Sirkka-Liisa Kanervo, Vuokko Liukkonen, and Eero Mattila for excellent technical assistance.

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Received for review September 1, 2005. Revised manuscript received November 11, 2005. Accepted November 16, 2005. The Finnish malting and brewing industry, the National Technology Agency of Finland, the Academy of Finland (Project 106528), the Raisio Group Research Foundation, and the Scientific Foundation for Women are acknowledged for financing the work.

JF0521505