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Enzymatic Hydrogenation of *trans*-2-Nonenal in Barley

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Conversion of undesirable, taste-active compounds is crucial for using barley as a suitable raw material for beer production. Here, ALH1, a barley alkenal hydrogenase enzyme that reduced the α,β-unsaturated double bond of aldehydes and ketones, was found to convert *trans*-2-nonenal (T2N), a major contributor to the cardboard-like flavor of aged beer. Although the physiological function of ALH1 in barley development remains elusive, it exhibited high specificity with NADPH as a cofactor in the conversion of several oxylipins—including T2N, *trans*-2-hexenal, traumatin, and 1-octen-3-one. ALH1 action represents a previously unknown mechanism for T2N conversion in barley. Additional experimental results resolved the genomic sequence for barley ALH1, as well as the identification of a paralog gene encoding ALH2. Interestingly, T2N was not converted by purified, recombinant ALH2. The possibility to enhance ALH1 activity in planta is discussed—not only with respect to the physiological consequences thereof—but also in relation to improved beer quality.

KEYWORDS: Malt; beer; taste stability; alkenal hydrogenase; nonenal; 1-octen-3-one

INTRODUCTION

Beer is one of the most widely consumed products in the beverage market, motivating brewers to perfect beer quality and taste stability. Better knowledge of brewing procedures, bottle materials, and storage conditions has reduced the effect of beer aging and allowed longer shelf life of the packaged product. However, for example, during distribution in warm climates, adequate storage conditions of beer cannot always be followed.

trans-2-Nonenal (T2N), which has an extremely low taste threshold [0.11 ppb; (*1*)], is widely considered to be a major contributor to the unpleasant cardboard flavor in aged beer (*2*). Only a few days at 38 °C suffice to increase the concentration of the aldehyde above taste threshold (*3*), but exactly how it is formed in the beer remains elusive. Whereas linoleic acid and its trihydroxy derivatives have been described to be transformed into T2N during heating at low pH (*4*, *5*), there is still no direct evidence that these reactions occur in a beer during normal aging. Recent findings suggested that T2N was released in beer by a nonoxidative mechanism (*6*), with the aldehyde derived from a precursor consisting of T2N bound to a nitrogenous compound (*7*).

Over the past decade numerous publications have focused on alternative ways to resolve the beer aging issue. Sulfite has received particular attention because it protects beer by forming nonvolatile, flavor-inactive adducts with several of the released aldehydes during aging, including T2N (*8*). Accordingly, a longer shelf life can be achieved in the presence of additional sulfite, either produced by yeast during fermentation or added to the finished beer before packaging. It is generally acknowledged that beer quality also is dependent on intrinsic properties of the barley (*Hordeum vulgare*) used to make the malt, a property that opens up the possibility to explore new targets that affect beer aging. Provided that the T2N precursor is a nitrogenous adduct formed through a reaction with the corresponding malt-derived aldehyde, then blocking the synthesis of T2N in the malt could improve the aging characteristics of the corresponding beer.

Plants degrade linoleic acid through the action of lipoxygenase (LOX) pathway enzymes, resulting in the generation of T2N and other oxylipins, a collective term for lipid- or fatty acid-derived molecules involved in plant defense, gene regulation, and plant-to-plant signaling (*9*). The enzymes and branches of the pathways are widely known in several plants including alfalfa and cucumber. Here, the reactions of the branch leading to T2N have been established (*10*, *11*). It was found to be generated from linoleic acid by two consecutive reactions, first catalyzed by a LOX enzyme and subsequently by a 9-hydroperoxide lyase (9-HPL). Whereas 9-LOX oxidized linoleic acid to 9-hydroperoxyoctadecadienoic acid (9-HPODE), 9-HPL cleaved the hydroperoxylinoleic acid to 9-oxononanoic acid and *cis*-3-nonenal, with the latter aldehyde further isomerized to T2N. Although little is known about the synthesis of T2N in barley, three LOXs have been identified in kernels (*12*). LOX-1 and LOX-2 predominantly catalyze the formation of 9-HPODE and 13-HPODE, respectively, whereas the specificity of LOX-3 remains elusive. 9-HPL activity has also been demonstrated in barley (*13*), but data on the corresponding gene and protein sequences are lacking. Recently, null-LOX-1 barley mutants were described (*14*, *15*), with the corresponding beer produced thereof reported to have improved taste stability and reduced levels of T2N, again implying that products of the LOX pathway affect beer quality. This discovery should not only stimulate further studies on the biological generation of T2N but also pave

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the way to improved taste stability by manipulating the enzymatic conversion of the aldehyde in barley or malt.

T2N belongs to the group of α,β-unsaturated aldehydes, which can react with nucleic acids and proteins to generate mutations and inactivation of enzymes (*16*). Consequently, organisms have evolved enzymatic ways for aldehyde detoxification, some of which have been elucidated: Glutathione (GSH)-*S*-transferases catalyze the conjugation of aldehydes with GSH (*17*), and aldo-keto reductases reduce aldehydes to the corresponding alcohols (*18*), whereas alcohol dehydrogenases oxidize aldehydes to the corresponding carboxylates (*19*). Recently, Mano et al. (*20*) identified an enzyme in *Arabidopsis thaliana* that catalyzed hydrogenation of the carbon−carbon double bond of α,β-unsaturated aldehydes. The enzyme was denoted alkenal hydrogenase (ALH; EC 1.3.1.74) and found to have exceptionally high affinity for T2N, a property indicating to us a direct link between a similar enzymatic activity and T2N levels in barley.

In analogy with the data described above, we suggest that conversion and/or degradation of T2N in barley can be enhanced such that less T2N will end up in the beer. To date, however, there is no direct evidence pointing to a specific barley enzyme with capacity to convert T2N. With that in mind, the aim of the present study was to establish whether barley synthesizes ALH orthologs, possibly followed by an enzymic characterization.

MATERIALS AND METHODS

Chemicals. All reagents employed in this study were obtained from commercial sources and used without further purification. T2N, *trans*-2-hexenal (T2H), and 1-octen-3-one were purchased from Sigma Aldrich, whereas traumatin and 12-oxophytodienoic acid (OPDA) were from Larodan (Malmö, Sweden). For nuclear magnetic resonance (NMR) analysis, deuterated chloroform ($CDCl_3$, 99.8% D) was purchased from Dentero (Heresback, Germany).

Nucleic Acids. Genomic DNA (gDNA) of *H. vulgare* cv. Barke was extracted using the Plant DNA Isolation kit (Roche). Using the *A. thaliana* ALH protein sequence as query [(*20*); GenBank accession number CAA89838], a search for homologues was done in the database of *H. vulgare* expressed sequence tags (ESTs) at The Institute for Genomic Research (TIGR), using the BLAST algorithm (*21*). Two tentative consensus sequences containing open reading frames (ORFs) encoding proteins with high identity to the *A. thaliana* ALH were identified. Subsequent sequence alignments were made using the blosum62mt2 scoring matrix with the ALIGNX software (Vector NTI, InforMax).

The DNA fragment specifying the ORF for ALH1 was amplified by PCR from gDNA of *H. vulgare* with the forward primer P1, CAT**ATG**GCGGCGGCGGCGGAGGTGGGCAACAG (*Nde*I site underlined; start codon in bold letters), and the reverse primer P2, GGATCC**TCA**CTCCCGTGCGACGGCCACCAGCTG (*Bam*HI site underlined; stop codon in bold letters). Cycling parameters were 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, for a total of 30 cycles. Following amplification of the 1864-bp genomic fragment, it was inserted into vector pCR2.1 TOPO (Invitrogen), resulting in plasmid pCR-ALH1, and subsequently sequenced using the MegaBace 9600 sequencer (Amersham Bioscience). The entire DNA sequence spanning five exons and four introns is available in the GenBank library under the accession number AY904340.

In parallel running reactions, the DNA fragment for the ORF specifying ALH2 was amplified by PCR from gDNA of *H. vulgare*. Here, reactions using the forward primer P3, CAT**ATG**GCGGAGCTCAAGAGCCGGCGGGTG (*Nde*I site underlined; start codon in bold letters), and reverse primer P4, GGATCC**TCA**CTCGGTGTCGGGGGTGGTGAGCTTG (*Bam*HI site underlined; stop codon in bold letters), yielded a 1062-bp fragment. This was inserted into vector pCR2.1 TOPO yielding pCR-ALH2, and subsequent sequencing revealed that it contained the entire ORF, but was without introns.

To construct an uninterrupted ORF encoding ALH1, each of the five exons—including 20-bp overhangs with the neighboring exon—were amplified by PCR using the following set of primers (forward primer followed by reverse primer): P1 and ACGCCCAGAGTGGTTAACACCTCCCCCTGGACGAAGTCCG for exon 1, CGGACTTCGTCCAGGGGGAGGTGTTAACCACTCTGGGCGT and GGCAGTAAGGCCAGGCATGCCAAGAACTCCTGTGTAGTAT for exon 2, ATACTACACAGGAGTTCTTGGCATGCCTGGCCTTACTGCC and TTTGTTTTTAGGAGGTTGACCTTCTCATCGGAACCGGCAC for exon 3, GTGCCGGTTCCGATGAGAAGGTCAACCTCCTAAAAACAAA and CGATGCCCTCCGGGAAGCACCTCTTCAGTGTGGCGTTCAG for exon 4, and CTGAACGCCACACTGAAGAGGTGCTTCCCGGAGGGCATCG and P2 for exon 5. The amplified fragments representing exons 1 and 2 were fused using splicing by overlap extension [SOE (*22*)] and subsequently fused with exon 3, etc. Eventually, the full-length fragment was inserted into pCR2.1 TOPO, yielding plasmid pCR-ALH1, and the entire sequence was verified, including the flanking sites for *Nde*I and *Bam*HI.

Total RNA was extracted from embryos of germinating kernels following instructions of the FastRNA Pro Green kit (Bio101 Systems).

Synthesis of Recombinant ALH1 and ALH2. The *Nde*I−*Bam*HI fragments consisting of the cDNA sequences for ALH1 and ALH2 were inserted into vector pET19b (Novagen) yielding plasmids pET19b-ALH1 and pET19b-ALH2, respectively. The ligation introduced the DNA code for MGHHHHHHHHHHSSGHIDDDDKH immediately upstream of the start codons for either protein. Next, *Escherichia coli* BL21(DE3)pLysS cells, purchased from Novagen, were transformed with the expression plasmids.

His-tagged recombinant ALH1 and ALH2 were expressed and purified as follows. Separate flasks containing 250 mL of Luria−Bertani broth, supplemented with 100 mg L^{-1} ampicillin, were inoculated with bacteria harboring pET19b-ALH1 or pET19b-ALH2 and propagated at 37 °C on a rotary shaker. After 1 h, protein expression was induced by adding 1 mM IPTG. The cultures were incubated overnight before cells were collected by centrifugation, then resuspended in 12.5 mL of BugBuster HT (Novagen), and lysed by incubation for 20 min at room temperature. Cell debris was pelleted by centrifugation, and the supernatants containing the recombinant proteins were further purified by Ni−HiTrap column chromatography according to established procedures (Amersham Biosciences). Elution of bound protein was done with a 0.1 M sodium phosphate buffer, pH 6.5, supplemented with 500 mM imidazole. The purity and concentration of eluted proteins was analyzed by sodium dodecyl sulfate−polyacrylamide gel eletrophoresis (SDS-PAGE) using molecular mass standards at known concentrations. When stored at 8 °C, the proteins remained fully active for at least 2 weeks.

Activity of the Purified Enzyme. ALH activity was measured by adding 2 μg of the purified enzyme to 2.5 mL of a 0.1-M potassium phosphate buffer, pH 6.6, supplemented with 1 mM NADPH and various concentrations of electron acceptors, that is, substrates (T2H, T2N, 1-octen-3-one, traumatin). These were dissolved in ethanol such that the resulting alcohol concentration was kept under 1% in the reaction buffer. Oxidation of NADPH was followed by a decrease in A_{340} using a spectrophotometer (Lambda 16; Perkin-Elmer). Initial reaction velocities were determined for the various substrate concentrations, with the kinetic constants K_m and V_{max} calculated using the Michaelis−Menten equation.

Synthesis of Aldehyde Conjugates. Dinitrophenylhydrazine (DNPH) conjugates were synthesized by combining 200 μL of pure aldehyde (1 mM), or ALH1 reaction product, with 250 μL of DNPH (0.2 M) and 50 μL of concentrated HCl. Following incubation in the dark at 37 °C for 1 h, the derivatized compounds were extracted twice with 1 mL of hexane. The combined hexane fractions were dried under N_2, followed by resuspending the precipitate in 100 μL of acetonitrile.

Measurement of Oxylipins. High-pressure liquid chromatography (HPLC) analysis was done on a HP-1100 apparatus (Hewlett-Packard), equipped with a 4.6 × 250 mm column (5-μm Symmetry C18, Waters). Chromatography was performed with the following gradient solvent system: 40% water (solvent A) and 60% acetonitrile (solvent B) for 15 min, ramped to 70% solvent B over 5 min, and then to 80% B over

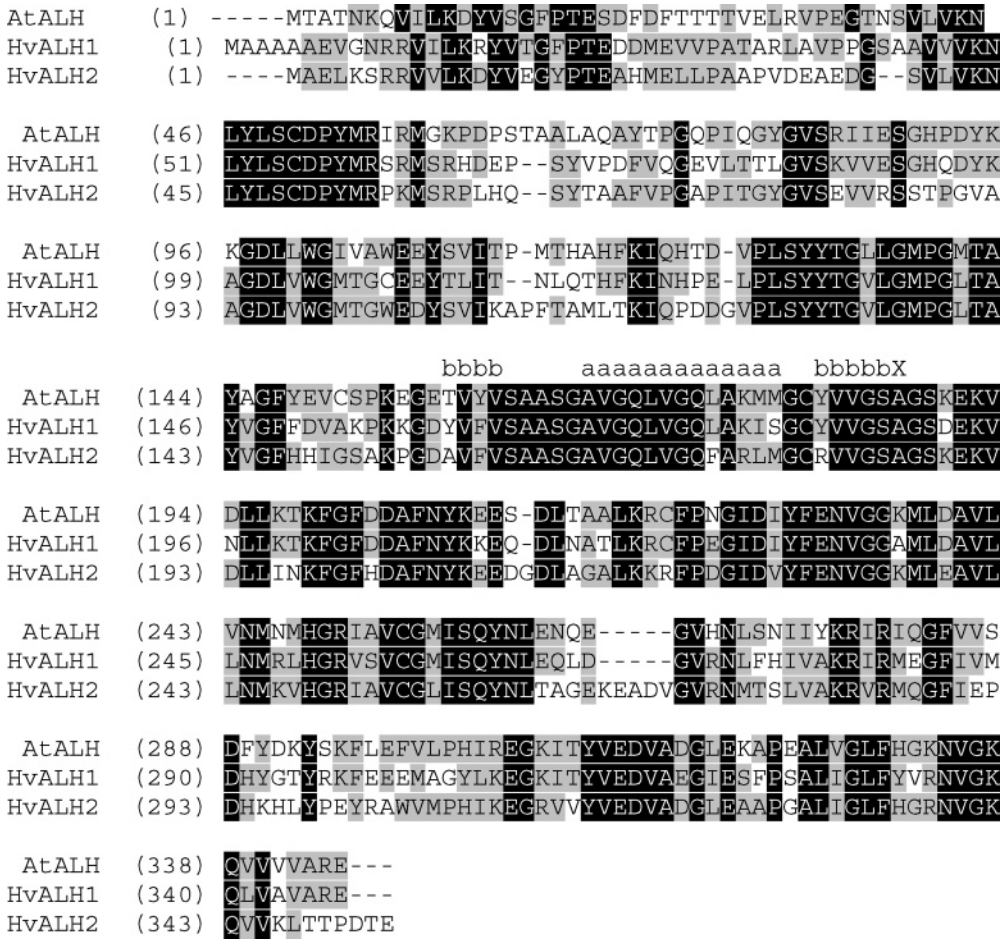


Figure 1. Alignment of ALH homologous sequences from *A. thaliana* (AtALH) and *H. vulgare* (HvALH1 and HvALH2). Identical amino acids are shaded in black and similar residues in gray. The predicted secondary structure of the Rossman fold, based on the *E. coli* QOR structural data (*26*), is marked with a or b to indicate α-helixes and *β*-strands, respectively. The putative NADPH/NADH discriminating residue is marked with X.

the next 15 min. The flow rate was 1.5 mL min^{-1}, and A_{365} of the effluent was monitored.

Product Analysis by NMR. All of the ^1H NMR spectra were recorded at 37 °C in solutions of $CDCl_3$ on a Bruker DRX-250 Avance spectrometer. A total of 128 scans were made. Spectral width of 5.2 kHz and collection of 32K data points were recorded with water during 2-s relaxation delays. The free induction decays (FID) were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor. The spectra were referenced against the residual signal of $CHCl_3$ at 7.27 ppm.

Extraction of Barley Enzymes. Barley enzyme extracts, each of three 2-day-old germinating kernels, were obtained by homogenizing the tissues in 500 μL of water supplemented with 10 μL of 10% (v/v) Triton-X. The suspension was incubated for 15 min at 4 °C and then centrifuged, and the resulting supernatant was stored at 4 °C.

RESULTS

Cloning and Sequence Analysis of *alh1*. Searches with the *A. thaliana* ALH sequence revealed two matches in the TIGR barley EST database, corresponding to the tentative consensus sequences TC140031 and TC131828. These consisted of 1044- and 1062-bp-long ORFs encoding proteins with 62.3 and 58% identity to the query, respectively (**Figure 1**). Both ORFs have a typical monocot-specific sequence around the putative start codon (23). In addition, the 1044-bp-long ORF is preceded by two, in-frame, stop codons. Using specific primers, either ORF was amplified from barley gDNA, resulting in fragments of 1864 bp for *alh1* and 1062 bp for *alh2*. Sequence analysis revealed that *alh2* contained a full-length ORF, whereas that for *alh1* defined a gene structure with five exons and four introns. The G-C contents of these exons and introns were 59.1 and 40.7%, respectively, in accordance with numerous plant genes. BLAST searches in the Gramene database (*24*) identified an *alh1* ortholog on chromosome 12 of rice, characterized by four introns at identical, relative positions as those of barley *alh1*. An *alh2* ortholog was identified on chromosome 4 in rice. Sequence alignment of ALH1 and ALH2 with their respective orthologs in rice showed that the proteins share 72.8 and 59.5% identity, respectively.

On the basis of sequence similarities, ALHs belong to the leukotriene B4 dehydrogenases (LTBDH) family of in the medium-chain dehydrogenases and reductases (MDR) super family (*25*). Several of the enzyme members, for example, alcohol dehydrogenases—but not those of the LTBDH family—contain one or two Zn atoms. In general, the MDR enzymes are predicted to share an N-terminal catalytic domain and a C-terminal nucleotide-binding domain, where the latter is characterized by the *β*,α,*β* motif known as the Rossman fold [(*26*); **Figure 1**].

MDR enzymes utilize either NAD(H) or NADP(H) as cofactor, a property partially conferred by the sequence motif GxGxxG/A (G, Gly; A, Ala; x, any amino acid) in the Rossman fold and the charge of the residue at the end of the second *β*-strand in the nucleotide-binding domain (*26*). In this respect, substituting the negatively charged Asp-223 residue with glycine in yeast alcohol dehydrogenase was found to increase its specificity for $NADP^+$. However, because the enzyme still could

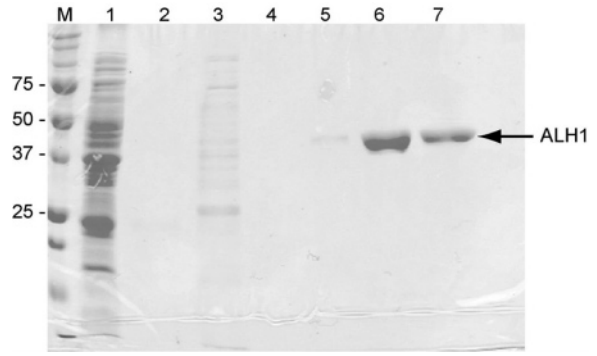


Figure 2. SDS-PAGE. Affinity purification of His-tagged ALH1 protein. Proteins bound to the affinity column were eluted and collected in a 1-mL fraction. Ten-microliter aliquots thereof were separated on 12% SDS-PAG. Elutions were with 20 mM imidazole (lanes 1 and 2), 120 mM imidazole (lanes 3 and 4), and 500 mM imidazole (lanes 5–7). M, molecular mass standards (in kDa). The horizontal arrow points to stained ALH1 protein.

reduce NAD^+, it was suggested that other residues contribute to its substrate specificity. The corresponding motif in barley ALH1 and ALH2, AxxGxxG, is identical to that of *E. coli* quinone oxidoreductase (QOR) and ALH of *A. thaliana*, each requiring NADPH for activity (*20*, *26*). Moreover, the proposed cofactor-determining residue, at the end of the second β-strand, is uncharged in both ALH1 and ALH2 (Ala-189 and Ala-186), suggesting that enzymic action depends on the availability of NADPH (**Figure 1**).

Recombinant ALH1 and ALH2. Heterologous expression of ALH1 and ALH2 was achieved in *E. coli* cells. To allow bacterial expression of ALH1, the introns of the genomic sequence were removed by SOE-PCR to yield a 1044-bp ORF. This and the 1062-bp *alh2* ORF were separately ligated downstream of a stretch for 10 His residues to simplify subsequent purification of the expressed proteins. *E. coli* BL21-(DE3)pLysS cells, transformed with the resulting constructs, were propagated according to standard procedures, followed by extraction of the recombinant enzymes. The homogenities of affinity-purified ALH1 and ALH2 were examined by SDS-PAGE, which showed single, ~38-kDa protein bands (**Figure 2**).

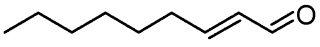
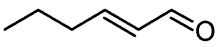
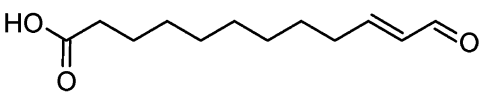
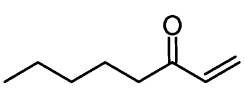
T2N Is a substrate for ALH1, Not for ALH2. In a first series of analyses, combinations of ALH1, NADPH, and T2N were followed spectrophotometrically at 340 nm to determine possible enzyme-catalyzed conversion of the cofactor. On the basis of these experiments, it was established that ALH1 reduced T2N rapidly with very high affinity, $K_m = 1.0$ μM (**Table 1**). When the cofactor NADPH was replaced with NADH, no conversion was observed, thus verifying the presumption that ALH1 action is NADPH-dependent. The highest conversion rate of T2N was measured at pH 6.5.

Unlike ALH1, attempts to obtain ALH2 action with NADPH and T2N caused no oxidation of the cofactor, raising the possibility of different substrate profiles for the paralogs. Additional electron acceptors were therefore tested as substrates for ALH2. One, traumatin, was shown to be reduced by the enzyme with a K_m of 110 μM. Although this demonstrated that heterologously expressed ALH2 was active, further studies of ALH2 action were beyond the scope of this work, as T2N remained inaccessible as a substrate. No ALH2-catalyzed reduction of traumatin was observed when NADPH was replaced with NADH.

ALH1 Catalyzes Hydrogenation of the Carbon−Carbon Double Bond of T2N. The reaction product of ALH1-catalyzed conversion of T2N was first examined by HPLC analysis. To facilitate handling and detection of the reaction compounds, volatile aldehydes were reacted with DNPH to form stable conjugates that are characterized by good light absorption properties. Reaction mixtures of T2N, NADPH, and ALH1 were incubated for 10 min at room temperature, then derivatized with DNPH, and subsequently examined by HPLC analysis (**Figure 3**). Derivatized, pure T2N was found to elute from the C18 column in two peaks with retention times of 33.0 and 34.1 min, primarily because isomeric products were obtained by isomerization with DNPH. However, the reaction product eluted as a single peak at 35.8 min, corresponding to that of derivatized nonanal. Accordingly, the experiment established that ALH1 catalyzed the expected reduction of T2N to nonanal. The reaction was enzymatic, as no conversion of T2N was observed when heat-inactivated ALH1 replaced active enzyme (data not shown).

NMR analysis was employed to experimentally assess the identity of the enzymatic product of ALH1 action. Nonanal, T2N, and T2N supplemented with ALH1 were combined in separate tubes with buffer and NADPH. After incubation overnight at room temperature, the reaction mixtures were extracted with $CDCl_3$. Examination of ^1H NMR spectra of the

Table 1. Structures of the Compounds Tested for Electron Acceptors and Kinetic Constants for Recombinant ALH1 Determined at 30 °C and pH 6.6 Using NADPH as Cofactor

Substrate	Structure	V_{max} (μM min^{-1} $μg^{-1}$)	K_m (μM)	Specific activity V_{max}/K_m (min^{-1} $μg^{-1}$)
T2N		1.6	1.0	1.6
T2H		0.65	10	0.065
Traumatin		1.3	0.8	1.6
1-Octene-3-one		0.8	2.0	0.4

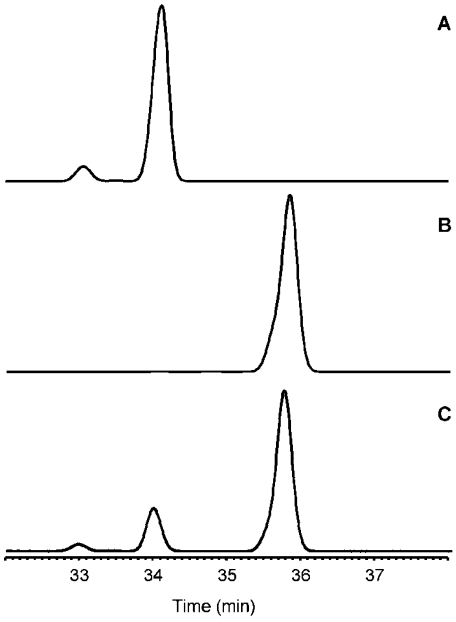


Figure 3. HPLC analysis of aldehydes. DNPH-derivatized T2N eluted as two peaks at 33.0 and 34.1 min (**A**), whereas DNPH-derivatized nonanal eluted at 35.8 min (**B**), identical to that of the derivatized reaction product from ALH1-catalyzed reduction of T2N (**C**).

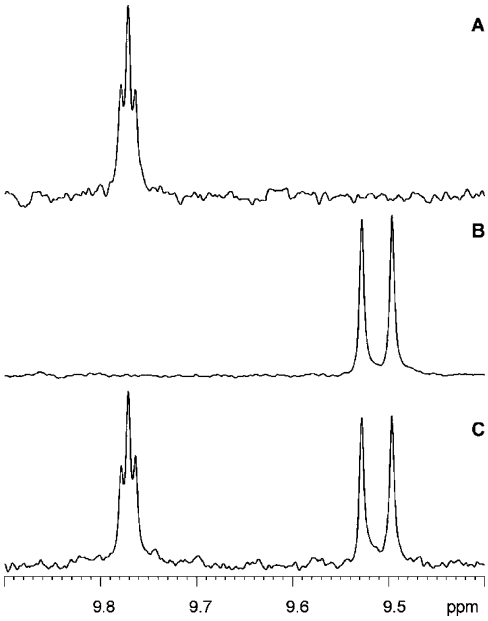


Figure 4. NMR analysis. 250 MHz ^{1}H NMR spectra of aldehydes extracted from reactions consisting of pure nonanal (**A**), pure T2N (**B**), and T2N and enzyme (**C**).

extracted aldehydes revealed that the product of enzyme-converted T2N was identical to nonanal and that NADPH alone could not reduce T2N. **Figure 4** shows the signals of the two different aldehyde protons at 9.77 ppm (triplet, $^3J_{H,H} = 1.6$ Hz, assigned to H-1 of nonanal) and 9.51 ppm (doublet, $^3J_{H,H} = 7.8$ Hz, H-1 of T2N). Other informative signals were those of the methylene protons in the trans double bond in T2N at 6.85 and 6.13 ppm (not shown).

Substrates. Instead of a full examination on potential substrates for ALH1 action, emphasis was on key intermediates of the LOX pathway. Here, T2H and traumatin represented two α,*β*-unsaturated aldehydes generated by 13-HPL-catalyzed cleavage of 13-HPODE (*27*). Whereas T2H has been suggested to function as an attractant for herbivore predators and as an antimicrobial substance (*28*, *29*), traumatin is considered to be a growth-stimulating agent formed in response to plant tissue damage (*30*). Kinetic analyses using recombinant ALH1 revealed that the enzyme reacted quickly with T2H and traumatin to form hexanal and 12-oxododecanoic acid, respectively. Although the affinity for traumatin was similar to that of T2N (0.8 μM), reactions with T2H yielded a >10-fold higher value ($K_m = 10$ μM; **Table 1**).

Finally, an interesting aroma compound, 1-octen-3-one, was tested as an electron acceptor for ALH1. The ketone is characterized by having a double bond between the α and *β* carbons, and a mushroom-like flavor with a taste threshold of 0.025 ppb, which is one-fifth of that for T2N (*1*). In the context of beer production, infections of malt with fungi may generate 1-octen-3-one and spoil the beer product. Because both *A. thaliana* ALH and LTB4DH are known to reduce α,*β* double bonds of ketones (*1*, *20*, *31*), it was assessed whether 1-octen-3-one might be a substrate for an ALH1-catalyzed reduction. An experiment with a mixture consisting of 1-octen-3-one, NADPH, and ALH1 showed that the ketone was a good substrate for the enzyme ($K_m = 2.0$ μM; **Table 1**). This result makes it conceivable that ALH1 exhibits wide substrate specificity and hints at useful applications of the enzyme in the conversion of flavor-intense ketone/aldehyde compounds.

Aldehydes without an α,*β*-unsaturated bond—nonanal, octanal, and hexanal—were also tested as substrates, but ALH1 could not reduce these molecules. In addition, OPDA did not serve as a substrate for ALH1, suggesting that the enzyme cannot readily reduce α,*β*-unsaturated bonds of cycloalkones. In summary, ALH1 was shown to share enzymatic properties with *A. thaliana* ALH, properties that support the categorization of ALH1 as an alkenal hydrogenase.

***alh1* Expression and ALH Activity in the Barley Embryo.** The identification and characterization of the ALH1 enzyme prompted an examination of the expression of the corresponding gene in barley kernels during germination. The focus was on kernels not only because they are known to contribute to the generation of LOX pathway-specific aldehydes but also because germinating kernels are highly relevant for processes involved in beer making. In a first step, searches in the TIGR database revealed 25 ESTs with absolute identity to *alh1* mRNA. The ESTs were derived from various plant parts, including leaves, endosperm, germinating kernel, and embryo. Accordingly, we hypothesize that *alh1* expression is constitutive and not restricted to specific tissues or developmental stages.

RNA was extracted from embryos of germinating barley kernels that had been incubated for 2 days in Petri dishes supplemented with water. RT-PCR was employed to amplify possible *alh1*-specific transcripts. For that purpose, the forward and reverse primers were designed to anneal in exon 2 and exon 5, respectively, such that an amplified fragment derived from processed *alh1* mRNA would have a length of 410 bp, whereas possible contaminating gDNA would yield a fragment of 820 bp. The PCR reaction products were analyzed by agarose gel electrophoresis, revealing a DNA band of 410 bp, which was confirmed by sequencing to be specific for *alh1* (data not shown). This result revealed that *alh1* was expressed in germinating kernels and confirmed the annotations given in the TIGR barley EST database.

To determine whether ALH1 contributes to the conversion of T2N in germinating barley kernels, aliquots of an embryo extract of water-soluble proteins were supplemented with combinations of T2N and NADPH, followed by incubation for 5 h at room temperature. However, although much of the T2N

was converted during the incubation, no traces of nonanal could be detected by the methods applied (data not shown). It remains unclear whether the majority of the T2N is converted by enzymes other than ALH1 or, alternatively, nonanal is a short-lived intermediate.

DISCUSSION

T2N is widely considered to be an important aldehyde that contributes to the stale flavor of aged beer. However, despite extensive analyses of the chemical reactions involved in the release of T2N in beer, its immediate precursor remains unknown. This lack of information has limited the development of new ways to prolong beer taste stability as alternatives to the conventional addition of sulfite. Nevertheless, the T2N-generating pathway in malt has been studied, eventually resulting in the identification of the LOX-1 enzyme which catalyzes the conversion of linoleic acid into 9-HPODE, a precursor of T2N in plants. Recently, a barley mutant lacking LOX-1 activity was described, and beer made from the corresponding malt revealed improved properties (*14*, *15*). This finding led to the suggestion that enzymatic generation of the aldehyde in malt affects, in part, the taste stability of beer, possibly such that the T2N level in malt correlates with the corresponding potential of beer. Further physiological studies of the metabolism of T2N in barley and malt should therefore facilitate a more comprehensive approach to its elimination. The work presented here represents a first step in this direction.

Using sequence searches for similarity with the alkenal-converting enzyme of *A. thaliana* (ALH), we identified and characterized a barley gene encoding a T2N-converting enzyme denoted ALH1. This was shown to catalyze an efficient reduction of T2N to nonanal, which has a taste threshold of 0.018 ppm, >150 times higher than that of T2N (*1*). In extracts of embryos of germinating kernels we could demonstrate the presence of *alh1* transcripts. It is therefore possible that T2N hydrogenation is part of cellular mechanisms for adjusting aldehyde levels, a property expected to affect the level of T2N in malt.

To evaluate ALH1 activity, we determined the V_{max}, which is a measure of the maximal velocity of the catalytic reaction, and K_m, a measure of the affinity of the enzyme for the substrate (**Table 1**). Considering the tight binding between enzyme and T2N ($K_m = 1.0\ \mu M$) and a high ratio of V_{max}/K_m (1.6 min^{-1} μg^{-1}), it is likely that enhanced ALH1 activity in barley can be exploited to decrease the amount of T2N in the corresponding malt. Because this approach could result in reduced levels of T2N precursors in the corresponding beer, a prolonged shelf life seems to be achievable for the packaged product. It is possible that the addition of recombinant ALH1 to the mash could increase the rate of T2N conversion. However, this would represent an economically expensive solution, as NADPH is required for enzymic action. Utilization of malt with enhanced ALH1 activity represents an alternative approach.

To identify or generate novel barley plants with enhanced ALH1 activity, at least two approaches are possible: (i) screening of barley for high levels or activities of ALH1 or (ii) overexpression of the *alh1* gene in transgenic barley. A screening could be employed on either existing or mutagenized barley cultivars, but the approach seems to be practically applicable only for the identification of mutants with decreased ALH activity. Few, if any, mutations result in increased activity, making the screening for hyperactive ALH1 plants very laborious with an uncertain outcome. In contrast, overexpression of the *alh1* gene and the subsequent increase in total ALH1 activity is possible using a transgenic approach. However, the success of any attempt to manipulate ALH1 activity will depend on the enzyme's biological role in the plant.

The substrate(s) of highest relevance for barley ALH1 action in vivo remain(s) elusive. Because ALH1 reduces the carbon−carbon double bond of α,β-unsaturated aldehydes/ketones, the enzyme can be active in numerous metabolic reactions outside the context of T2N conversion, for example, in the reduction of OPDA in the LOX pathway (*9*) and in the conversion of campesterol to campestanol within the brassinolide synthesis pathway (*32*). However, OPDA did not serve as substrate for ALH1 (data not shown), indicating that the enzyme is incapable of reducing α,β-unsaturated bonds of cycloalkones, similar to that observed for *A. thaliana* ALH and LTBDH (*20*, *31*).

Other molecules identified as putative ALH1 substrates include T2H and traumatin, oxylipins that have been implicated in various stress responses in plants. Whereas T2H is known to possess antimicrobial properties (*29*) and may attract herbivore predators (*28*), the function of traumatin remains unknown, although it has been suggested to play a role in cell division processes (*30*). The function and metabolism of these two oxylipins are of particular interest, because they are synthesized in equimolar amounts upon cleavage of 13-HPODE by 13-HPL (*27*). Concomitant ALH1-catalyzed reduction of both T2H and traumatin to the corresponding saturated molecules is possible, but with substantially different kinetics. Because the ratio of V_{max}/K_m is ∼25-fold higher for traumatin than for T2H (**Table 1**), it is conceivable that ALH1 has a function in regulating the relative amounts of T2H and traumatin.

Plant-derived α,β-unsaturated aldehyde oxylipins are widely considered to be associated with various types of physical stress. The molecules are very reactive and have been reported to inactivate enzymes and induce mutations (*16*). Aldehyde detoxification is considered to be important, and several experiments have demonstrated a relationship between stress and expression of oxylipin-converting enzymes (*33*, *34*). Moreover, the overexpression of an aldehyde dehydrogenase in *A. thaliana* (*33*) and of an aldose/aldehyde reductase in tobacco (*35*) resulted in plants with improved stress tolerance. These data, and the fact that unsaturated aldehydes are much more toxic than the corresponding saturated molecules (*29*), suggest that barley ALH1 represents an enzyme that is actively involved in plant stress responses. Accordingly, overexpression of ALH1 in barley could have beneficial commercial effects, not only in brewing by decreasing the amount of T2N in the corresponding malt but also in breeding by improving the plant's stress tolerance.

A practical consideration in malting is the occasional infections of fungi that give the brew a mushroom-like flavor, which is attributed to the presence of the fungal fatty acid-derived metabolite 1-octen-3-one (*27*, *36*). Because ALH1 converts 1-octen-3-one to 3-octanone, a product with a 20000-fold higher taste threshold in beer than its corresponding substrate (*1*), it is possible that malt overexpressing *alh1* could suppress some adverse effects of fungal infections.

It is likely that barley expresses several enzymes with the ability to convert α,β-unsaturated aldehydes, a notion supported by the finding of four potential ALH1 orthologs in a BLAST search of the rice genome. The orthologs were found on chromosomes 1, 4, 11, and 12, with the respective identities to barley ALH1 being 56.7, 67.8, 63.8, and 72.7%. A barley paralog, ALH2, with 56.5% identity to ALH1, was identified in the TIGR barley EST database. Following cloning and characterization of the gene encoding ALH2, we discovered that the corresponding heterologous enzyme catalyzed the same

reaction as ALH1, that is, hydrogenation of the double bond of α,β-unsaturated aldehydes (data not shown). However, comparison of reaction kinetics between ALH1 and ALH2 revealed distinct differences. ALH2 could, for example, not convert T2N, leaving open the possibility that the ALH paralogs have different biological roles. So far, traumatin is the only substrate identified for ALH2, but a high K_m value (110 μM) suggests that other molecules exhibit more important biological roles in vivo.

It is likely that an examination of the regulation of the *alh* genes would provide important clues to the physiological role(s) of this gene family. In addition, studying barley plants with reduced or increased ALH activity could be helpful. In any case, the potential commercial value of such mutants should motivate both brewers and breeders to further study these genes and enzymes.

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LITERATURE CITED

(1) Meilgaard, M. C. Flavor chemistry of beer: part II. Flavor and threshold of 239 aroma volatiles. *MBAA Tech. Q.* **1975**, *12*, 151−168.

(2) Jamieson, A. M.; van Gheluwe, J. E. A. Identification of a compound responsible for cardboard flavor in beer. *Proc. Am. Soc. Brew. Chem.* **1974**, 192−197.

(3) Wang, P.; Siebert, K. Determination of *trans*-2-nonenal in beer. *MBAA Tech. Q.* **1974**, *11*, 110−117.

(4) Drost, B. W.; van Eerde, P.; Hoekstra, S. F.; Strating, J. Fatty acids and staling of beer. *EBC Proc.* **1971**, 451−458.

(5) Witt, P. R.; Burdick II, A. Lipids in brewing and brewing materials. *ASBC Proc.* **1961**, *19*, 104−110.

(6) Lermusieau, G.; Noel, S.; Liegeois, C.; Collin, S. Nonoxidative mechanism for development of *trans*-2-nonenal in beer. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 29−33.

(7) Liegeois, C.; Meurens, N.; Badot, C.; Collin, S. Release of deuterated (*E*)-2-nonenal during beer aging from labeled precursors synthesized before boiling. *J. Agric. Food Chem.* **2002**, *50*, 7634−7638.

(8) Nyborg, M.; Outtrup, H.; Dreyer, T. Investigations of the protective mechanism of sulfite against beer staling and formation of adducts with *trans*-2-nonenal. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 24−28.

(9) Feussner, I.; Wasternack, C. The lipoxygenase pathway. *Annu. Rev. Plant Biol.* **2002**, *53*, 275−297.

(10) Noordermeer, M. A.; Veldink, G. A.; Vliegenthart, J. F. Alfalfa contains substantial 9-hydroperoxide lyase activity and a 3*Z*: 2*E*-enal isomerase. *FEBS Lett.* **1999**, *443*, 201−204.

(11) Matsui, K.; Ujita, C.; Fujimoto, S.; Wilkinson, J.; Hiatt, B., et al. Fatty acid 9- and 13-hydroperoxide lyases from cucumber. *FEBS Lett.* **2000**, *481*, 183−188.

(12) van Mechelen, J. R.; Schuurink, R. C.; Smits, M.; Graner, A.; Douma, A. C., et al. Molecular characterization of two lipoxygenases from barley. *Plant Mol. Biol.* **1999**, *39*, 1283−1298.

(13) Kuroda, H.; Furusho, S.; Maeba, H.; Takashio, M. Characterization of factors involved in the production of 2(*E*)-nonenal during mashing. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 691−697.

(14) Hirota, N.; Kaneko, T.; Kuroda, H.; Takoi, K.; Takeda, K. Barley lipoxygenase 1 gene, method of selecting barley variety, material of malt alcoholic drinks and process for producing malt alcoholic drink. PCT Int. Appl. WO04/085652, 2004.

(15) Hirota, N.; Kuroda, H.; Takoi, K.; Kaneko, T.; Kaneda, H., et al. Brewing performance of lipoxygenase-1-less barley. *Brew. Dig.* **2004**, 35.

(16) Witz, G. Biological interactions of α,β-unsaturated aldehydes. *Free Radical Biol. Med.* **1989**, *7*, 333−349.

(17) Hubatsch, I.; Ridderstrom, M.; Mannervik, B. Human glutathione transferase A4-4: an α class enzyme with high catalytic efficiency in the conjugation of 4-hydroxynonenal and other genotoxic products of lipid peroxidation. *Biochem. J.* **1998**, *330*, 175−179.

(18) Burczynski, M. E.; Sridhar, G. R.; Palackal, N. T.; Penning, T. M. The reactive oxygen species and Michael acceptor-inducible human aldo-keto reductase AKR1C1 reduces the α,β-unsaturated aldehyde 4-hydroxy-2-nonenal to 1,4-dihydroxy-2-nonene. *J. Biol. Chem.* **2001**, *276*, 2890−2897.

(19) Sellin, S.; Holmquist, B.; Mannervik, B.; Vallee, B. L. Oxidation and reduction of 4-hydroxyalkenals catalyzed by isozymes of human alcohol dehydrogenase. *Biochemistry* **1991**, *30*, 2514−2518.

(20) Mano, J.; Torii, Y.; Hayashi, S.; Takimoto, K.; Matsui, K., et al. The NADPH:quinone oxidoreductase P1-ζ-crystallin in *Arabidopsis* catalyzes the α,β-hydrogenation of 2-alkenals: detoxication of the lipid peroxide-derived reactive aldehydes. *Plant Cell Physiol.* **2002**, *43*, 1445−1455.

(21) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403−410.

(22) Horton, R. M.; Hunt, H. D.; Ho, S. N.; Pullen, J. K.; Pease, L. R. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **1989**, *77*, 61−68.

(23) Joshi, C. P.; Zhou, H.; Huang, X.; Chiang, V. L. Context sequences of translation initiation codon in plants. *Plant Mol. Biol.* **1997**, *35*, 993−1001.

(24) Ware, D.; Jaiswal, P.; Ni, J.; Pan, X.; Chang, K., et al. Gramene: a resource for comparative grass genomics. *Nucleic Acids Res.* **2002**, *30*, 103−105.

(25) Nordling, E.; Jornvall, H.; Persson, B. Medium-chain dehydrogenases/reductases (MDR). Family characterizations including genome comparisons and active site modeling. *Eur. J. Biochem.* **2002**, *269*, 4267−4276.

(26) Edwards, K. J.; Barton, J. D.; Rossjohn, J.; Thorn, J. M.; Taylor, G. L., et al. Structural and sequence comparisons of quinone oxidoreductase, ζ-crystallin, and glucose and alcohol dehydrogenases. *Arch. Biochem. Biophys.* **1996**, *328*, 173−183.

(27) Noordermeer, M. A.; Veldink, G. A.; Vliegenthart, J. F. Fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in wound healing and pest resistance. *ChemBioChem* **2001**, *2*, 494−504.

(28) Halitschke, R.; Ziegler, J.; Keinanen, M.; Baldwin, I. T. Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in *Nicotiana attenuata*. *Plant J.* **2004**, *40*, 35−46.

(29) Trombetta, D.; Saija, A.; Bisignano, G.; Arena, S.; Caruso, S., et al. Study on the mechanisms of the antibacterial action of some plant α,β-unsaturated aldehydes. *Lett. Appl. Microbiol.* **2002**, *35*, 285−290.

(30) Zimmermann, D. C.; Coudron, C. A. Identification of traumatin, a wound hormone, as 12-oxo-*trans*-10-dodecenoic acid. *Plant Physiol.* **1979**, *63*, 536−541.

(31) Dick, R. A.; Kwak, M. K.; Sutter, T. R.; Kensler, T. W. Antioxidative function and substrate specificity of NAD(P)H-dependent alkenal/one oxidoreductase. A new role for leukotriene B4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. *J. Biol. Chem.* **2001**, *276*, 40803−40810.

(32) Noguchi, T.; Fujioka, S.; Takatsuto, S.; Sakurai, A.; Yoshida, S., et al. *Arabidopsis det2* is defective in the conversion of (24*R*)-24-methylcholest-4-en-3-one to (24*R*)-24-methyl-5α-cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiol.* **1999**, *120*, 833−840.

(33) Sunkar, R.; Bartels, D.; Kirch, H. H. Overexpression of a stress-inducible aldehyde dehydrogenase gene from *Arabidopsis thaliana* in transgenic plants improves stress tolerance. *Plant J.* **2003**, *35*, 452−464.

(34) Kirch, H. H.; Nair, A.; Bartels, D. Novel ABA- and dehydration-inducible aldehyde dehydrogenase genes isolated from the resurrection plant *Craterostigma plantagineum* and *Arabidopsis thaliana*. *Plant J.* **2001**, *28*, 555−567.
(35) Oberschall, A.; Deak, M.; Torok, K.; Sass, L.; Vass, I., et al. A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. *Plant J.* **2000**, *24*, 437−446.
(36) Wurzenberger, M.; Grosch, W. Origin of the oxygen in the products of the enzymatic cleavage reaction of linoleic acid to 1-octen-3-ol and 10-oxo-*trans*-8-decenoic acid in mushrooms (*Psalliota bispora*). *Biochim. Biophys. Acta* **1984**, *794*, 18−24.

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