Ascorbic Acid Oxidase in Barley and Malt and Its Possible Role During Mashing[1]

Makoto Kanauchi, *Department of Food Management, Miyagi University, 2-2-1 Hatatate Taihaku-ku Sendai Miyagi, 982-0215, Japan;* **and Kyle J. Simon and Charles W. Bamforth,**[2] *Department of Food Science and Technology, University of California, Davis 95616, USA*

ABSTRACT

J. Am. Soc. Brew. Chem. 72(1):30-35, 2014

Ascorbic acid oxidase (AAO) develops in the embryo tissues of barley during steeping and initial stages of germination. Two AAO enzymes have been identified. One of them is of remarkably low molecular weight (<10,000). Both are very heat tolerant and capable of acting over a broad pH range. Both enzymes would be expected to function during conversion temperatures of mashing. Indeed, addition of ascorbic acid to mashes results in the survival of higher levels of polyphenol and thiols into wort and a reduced color in that wort, commensurate with AAO preferentially consuming oxygen which, thus, is less readily available for other reactions in mashes, including thiol oxidation and polyphenol oxidation.

Keywords: Ascorbic acid, Embryo, Heat resistant, Mashing, Molecular weight, Oxidase

RESUMEN

La oxidasa de ácido ascórbico (OAA) se desarrolla en los tejidos embriónicos de la cebada durante el remojo y las etapas iniciales de la germinación. Dos enzimas OAA han sido identificadas. Uno tiene un peso molecular inusualmente bajo (<10.000). Ambos son muy tolerantes al calor y son activas en un amplio rango de pH. Se esperaría que ambas enzimas funcionasen a las temperaturas de conversión del almidón durante la maceración. De hecho, la adición de ácido ascórbico a la mezcla resulta en la supervivencia de mayores niveles de polifenol y de tioles en el mosto, resultando en un color reducido, lo que concuerda con el hecho de que OAA consume oxígeno preferencialmente, lo que significa que el oxígeno está menos disponible a otras reacciones en la mezcla, incluyendo las oxidaciones de tiol y polifenol.

Palabras claves: Ácido ascórbico, Embrión, Maceración, Oxidasa, Peso molecular, Resistencia al calor

[1] Preliminary reports of aspects of this work were present at the World Brewing Congress, Portland OR, July/August 2012 and to the ASBC, Tucson, AZ, May, 2013

[2] Corresponding author. Phone: +1-530-752-9476; Fax: +1-530-752-4759; E-mail: cwbamforth@ucdavis.edu

http://dx.doi.org/10.1094/ASBCJ-2014-0120-01

Ascorbic acid oxidase (AAO; EC 1.10.3.3) has been known since 1931, when first identified (as "hexoxidase") in cabbage leaf (23). Since then, the enzyme has been widely reported in various plant and fungal tissues (Table I) but, as yet, its precise function is under debate (7). It catalyzes the reaction 2 L-ascorbate + $O_2 \rightarrow$ 2 dehydroascorbate + 2 H_2O (1).

Reports of AAO in barley are limited. Honda studied the enzyme in barley roots and found it to be associated with cell walls (9), in keeping with the findings of others (16). Tamas et al (24) studied the impact of cadmium (Cd) on AAO in germinating barley seed, with the agent causing substantial inhibition of rootlet growth. Cd inhibited two cationic AAO enzymes and also two anionic isozymes. A fifth AAO, this one cationic, was activated by Cd. Zelinova (25) also investigated the impact of Cd in inhibiting AAO.

Leaving aside the issue of its functionality in the economy of a growing plant, the question is begged of whether we can confirm the presence of such an enzyme in malt and what its significance might be in the context of brewing, which has not been hitherto explored. We have recently reported the existence in malt of a related enzyme, ascorbate peroxidase (12). It was shown that this peroxidase has a very high affinity for hydrogen peroxide and that the enzyme might have a valuable role in removing that reactive oxygen species; however, the enzyme is relatively heat sensitive and would not survive well in mashing scenarios.

We now report the presence of AAO and show that its properties are rather more commensurate with an impact on "oxygen economy" in sweet wort production.

EXPERIMENTAL

Preparation of Malt

Barley (100 g, 'Metcalf', harvested in Canada, 2008) was germinated according to Hoy et al (10). After treating the barley with a 1% sodium hypochlorite solution, it was washed with sterile water. Then, the barley was steeped in water at 16°C for 8 hr, before being drained and allowed to "air rest" at 16°C for 16 hr. The barley was resteeped in water at 16°C for 24 hr prior to germinating at 16°C for 6 days. The green malt was dried by lyophilization (Eyela FDU-2100; Tokyo Rikakikai Co. Ltd.).

TABLE I
Comparison of Ascorbic Acid Oxidases from Different Organisms[a]

Organism	Molecular Weight	pH Optimum	K_m Ascorbic Acid (m*M*)	K_m Oxygen (m*M*)	Heat tolerance	Inhibitors	Activators	References
Barley						Cadmium	Cadmium	24
Wheat	139,000 (dimer)	6.2	0.3	…	Stable at 40°C for 30 min	…	…	8
Maize		5.8	…	…	…	…	…	6
Squash	150,000 (dimer)	5.5 (max at 7.0); 6.0	0.2	…	Half life of 21 min at 70°C; destroyed in <1 min at 100°C	Azide, thiourea	…	3,20
Acremonium	80,000	4.0	0.29	0.47	Survives 30 min at 60°C	Azide, cyanide, Fe^{2+}, H_2S	Copper	11

[a] K_m (Michaelis constant) is the substrate concentration corresponding to half maximum velocity.

Preparation of Extracts

Barley or malt was ground in a blender (AS ONE Model 7011HS, catalog number 5340801) and extracted by stirring on ice in 2.5 vol of 2 m*M* EDTA in phosphate buffer (50 m*M*, pH 7.0) for 3 hr at 4°C. The slurry was then strained through cheesecloth and the resulting liquid was centrifuged at 10,000 × *g*. The resulting supernatant was referred to as "crude extract".

Endosperm Slices

Slices (2 mm) were cut from sterile, dehusked barley at a distance of 2 mm behind the scutellum (2). The slices were incubated at room temperature in sterile petri dishes (9 cm) containing gibberellic acid (10^{-5} *M*; Sigma-Aldrich, catalog number 7645) sterilized by filter (Minisart SPR15). After incubation, 5 mL of 50 m*M* phosphate buffer (pH 7.0) was added and the tissues were ground with a pestle and mortar. The homogenate was kept for 1 hr at room temperature before removal of particulate material by centrifugation at 10,000 × *g*.

AAO Assay

AAO activity was assayed on the basis of measuring the oxidation of ascorbate by the decrease in absorbance at 265 nm (e = 14 m*M* cm^{-1}) at 25°C (19). The reaction mixture contained 50 m*M* potassium phosphate buffer (pH 7.0), 0.5 m*M* ascorbate, and enzyme solution. One unit of enzyme catalyzes the oxidation of 1 m*M* ascorbic acid (AA) per minute.

AA Peroxidase Assay

AA peroxidase was assayed as described previously (12).

Optimum pH Determination

Optimum pH was evaluated over the pH range 2.0–10.0 by performing the assay using citrate-phosphate buffer (pH 4.0–5.0), phosphate buffer (pH 5.0–8.0), and borate buffer (pH 8.0–9.0).

Purification of AAO

Malt (100 g), milled as described earlier, was extracted for 3 hr at 4°C with three volumes of 50 m*M* citrate-phosphate buffer (pH 7.0) containing 2 m*M* EDTA and then centrifuged at 10,000 × *g* for 10 min. The enzyme extraction was applied to a column (25 by 300 mm) of Macro-Prep CM support (Bio-Rad; http://www.bio-rad.com/LifeScience/pdf/Bulletin_9292.pdf). The protein was eluted using a 0–1 *M* linear gradient of sodium chloride flowing at 1.5 mL min^{-1}. Fractions containing AAO were collected and reprecipitated using 80% saturation of ammonium sulfate. The precipitate was redissolved in 2 mL of 50 m*M* citrate-phosphate (pH 7.0), then applied to the size-exclusion column (10 by 350 mm, P-100 gel; Bio-Rad). The eluent was citrate-phosphate, 50 m*M*, pH 7.0 flowing at 2 mL min^{-1}.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The samples were separated on a 12.5% uniform gel (e-PAGEL, catalog number E-T12.5L; ATTO) or a 15% uniform gel (e-PAGEL, catalog number E-T15S; ATTO). Electrophoresis was carried out as described elsewhere (13,21), with the following modifications: the samples (0.01 mL) were added to 0.01 mL of sample buffer and then heated at 100°C for 3 min. Samples were added at 10 µL per well. The gels were run at 20 mA at a gel thickness of 1.5 mm. Molecular weight standards were from Bio-Rad Laboratories, Inc. The gel was stained with 0.25% Coomassie Brilliant Blue R-250.

Protein Concentration Determination

Protein concentration was determined using the method of Bradford (4). The standard curve was produced using bovine serum albumin (catalog number 05482; Sigma-Aldrich).

Location of AAO in Grain Using Stains

Barley or malt (1.0 g) was steeped in 1% AA solution in phosphate buffer (50 m*M*, pH 7.0) at 30°C for ≈1–4 hr. After reaction, residual AA was detected by 1 mL of 2,6-dichlorophenolindophenol solution (2.5 mg in 100 mL of deionized water), as described elsewhere (14).

Heat Stability Experiment

The enzyme solution was heated at 40–100°C for 30 min at pH 6.0 and cooled in ice prior to assay.

Determination of Kinetic Parameters

Two substrate kinetic analysis was conducted according to Dalziel (5) using a system as shown in Figure 1. Substrate solutions (1 mL, 40°C) containing 0.0625, 0.125, 0.25, 0.5, or 1.0 m*M* AA were introduced into the vial containing an electrode for measuring dissolved oxygen (TOKO Chemical Laboratory Co. Ltd.) and sealed with a rubber seal. A vacuum was drawn in the vial and nitrogen gas (99.9%; Tomoe Shokai Co.), oxygen gas (99%; Tomoe Shokai Co.), or nitrogen-oxygen mixed gases (20–80% oxygen in nitrogen gas) were successively flushed through the vial. The operation was repeated three times. Enzyme solution was added by a microsyringe through the seal. Decrease in absorbance at 265 nm of the solutions was measured using a Nano-Drop 2000 (Thermo Fisher Scientific).

Impact of AA Additions in Mashing

Pale Malt (two-row; Great Western Malting) was milled in a Miag mill (MIAG Braunschweig) on the coarse setting (0.7 mm). Mashes at 65°C were performed in a bath (Canongate CM3; Canongate Technology Ltd.) with 50 g of milled malt, 150 mg of AA, and 150 mL of deionized water. The mashes designated for 0 min were filtered immediately upon mixing and subsequent mashes were removed at 10, 20, 40, and 60 min. Once removed, mashes were filtered through Whatman 2555½, 320-mm cone filters into an ice bath. Wort samples were cooled to ≈4°C and

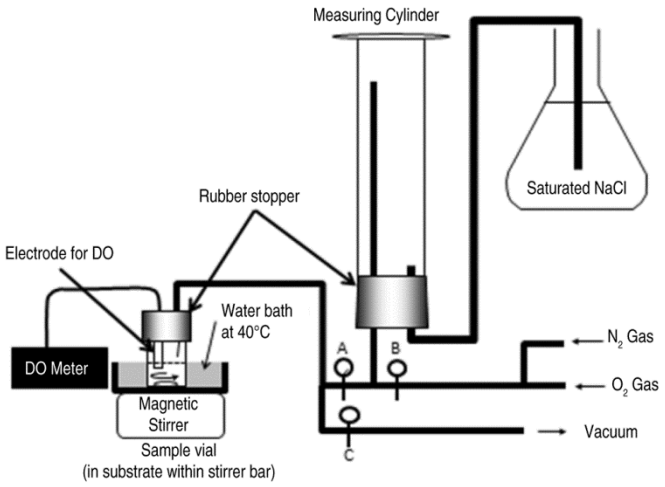

Fig. 1. Apparatus for determining kinetic parameters for ascorbic acid oxidase. Air is removed from the vial using vacuum with stopcock C open. To adjust oxygen concentration, a mixture of oxygen and nitrogen (e.g., 10 mL of O_2 and 90 mL of N_2) is transferred to the measuring cylinder filled with saturated NaCl solution via stopcock B. Upon closing B and opening stopcock A, the gas mixture is sucked vigorously to the vial from cylinder. The operation is repeated three times to achieve a stable oxygen content, as measured using the dissolved oxygen meter. Enzyme is added to the substrate mixture by microsyringe. After reaction, 10 µL of the vial contents are transferred by microsyringe for measurement of absorbance at 265 nm by Nano-Drop 2000.

analyzed as soon as possible. pH was measured using an Orion Research expandable ionAnalyzer EA 920 after manual inversion. After cone filtration, between 5- and 10-mL samples of wort were passed through glass fiber filters. The resulting sample was run through an Anton Paar DMA 4100m density meter to determine specific gravity. Color was determined by taking the absorbance at 430 nm of the remaining sample in a plastic cuvette (1). Total polyphenols were measured in accordance with the ASBC method (1). Free thiols were determined using the method of Muller (18).

RESULTS

Enzyme Properties

AAO is not present in ungerminated barley but starts to be synthesized immediately upon steeping (Fig. 2). It reaches a maximum level of activity early in germination, thereafter decreasing to a low but finite level at the end of germination. By contrast, ascorbate peroxidase is synthesized rather later.

AAO is primarily located in the embryo of dissected sprouted grain with successively less enzyme in the proximal and distal endosperm (*data not shown*).

Fractionation of crude extracts of malt by cation exchange chromatography revealed two peaks of AAO activity (Fig. 3), which are designated AAOI and AAOII. Further chromatography of these peaks on Bio-Gel P100 indicated that AAO1 was of higher molecular size than is AAOII (Fig. 4A and B), and this was confirmed by polyacrylamide gel electrophoresis (Fig. 5). Molecular weight estimates for the two enzymes are ≈25–27,000 and 6–9,000, respectively.

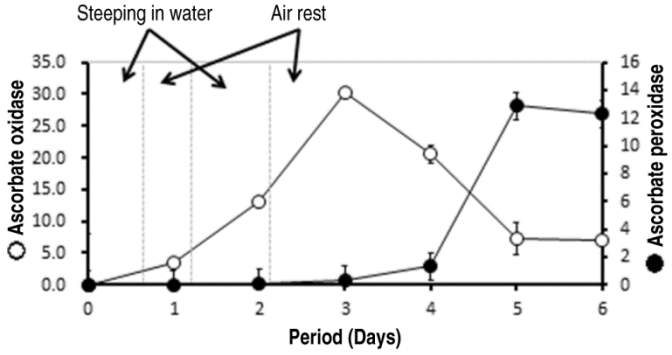

Fig. 2. Levels of ascorbic acid oxidase and ascorbate peroxidase during the steeping and germination of barley. The zero time point represents the barley prior to steeping. Enzyme values are quoted as units per gram of malt.

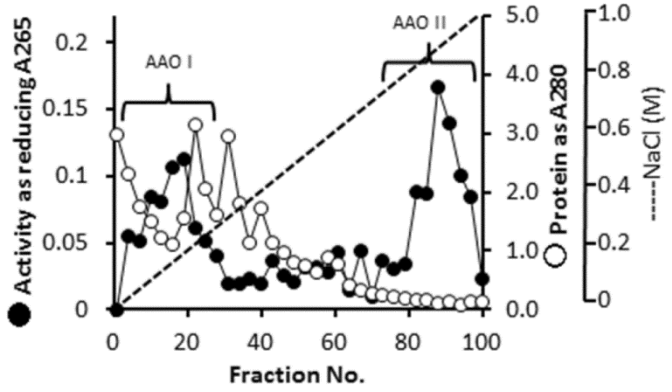

Fig. 3. Ion exchange chromatography of ascorbic acid oxidase (AAO).

Both enzymes had a pH optimum of ≈7 but AAOI has a broader activity range (Fig. 6). Both enzymes would be expected to display significant activity at mashing pHs and in beer.

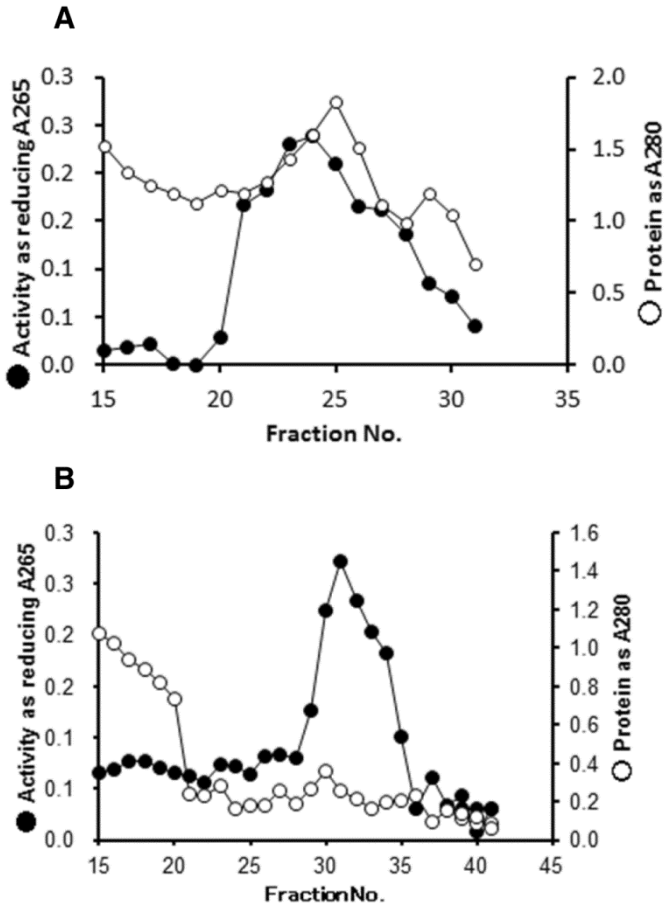

Fig. 4. Gel permeation chromatography of **A,** ascorbic acid oxidase I and **B,** ascorbic acid oxidase II.

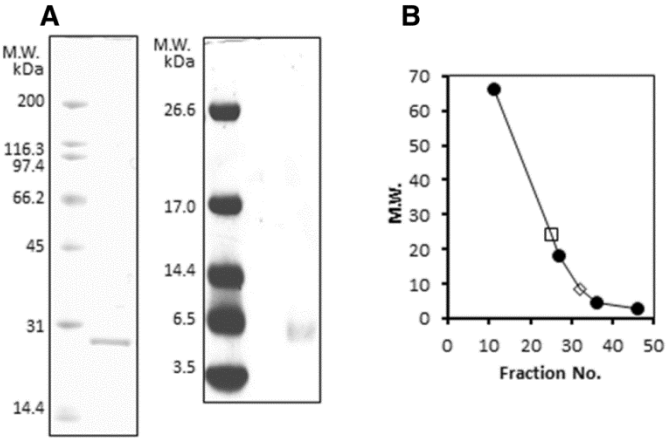

Fig. 5. Molecular weight determination for ascorbic acid oxidase. **A,** Sodium dodecyl sulfate polyacrylamide gel electrophoresis. The left-hand illustration represents ascorbic acid oxidase I (AAOI) and the right-hand one represents ascorbic acid oxidase II (AAOII). In each case, the lane to the left shows molecular weight standards and the right-hand lane the purified enzyme. **B**, By gel permeation chromatography. Filled circles indicate reference proteins; □ = AAOI and ◇ = AAOII.

Both enzymes are relatively heat tolerant (Fig. 7). Of the two, the very low molecular weight AAOII is really rather phenomenally thermotolerant, with some 20% of the activity surviving a 1-hr boil.

The enzyme may display slight activation by manganese and zinc; however, it is inhibited by copper, despite AAO in most plants generally being described as an enzyme rich in copper (Table II). Strong inhibition by EDTA and EGTA would be consistent with the need for a metal ion in the action of the enzymes, though iron and magnesium (and mercury) are potent inhibitors, especially of AAOII. Inhibition by azide suggests the presence of a functional heme group in the enzyme. Inhibition by iodoacetate indicates a functional presence of thiol groups. Inhibition by N-bromosuccinimide suggests a role for tryptophan, and inhibition by benzenesulfonyl fluoride would be consistent with a role for a serine group.

Two substrate kinetic analysis (Fig. 8A and B; Table III) revealed that AAOI is capable of operating faster than AAOII (higher true maximum velocity [V_{max}]). However, AAOII has a much greater affinity for both substrates (lower K_m values).

Mashing Studies

Mashes were performed at 65°C in either the presence or absence of 5.7 m*M* AA. This value is well in excess of the K_m value for AAOII, which, thus, would be expecting to operate at maximum rate at the start of mashing (Table IV). The presence of AA had little impact on the specific gravity of recovered worts. Unsurprisingly, it lowered the pH of the mash, although this rose progressively through mashing whereas the pH of the control

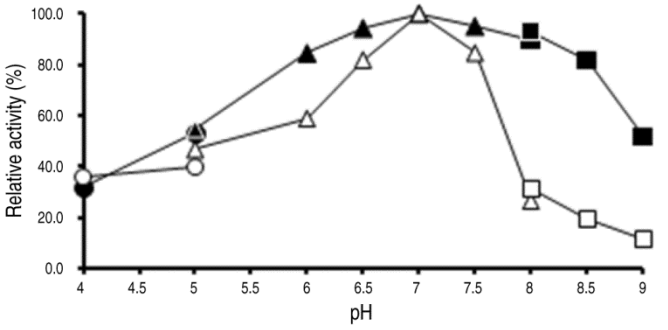

Fig. 6. pH optimum for ascorbic acid oxidase. Symbols: ○ and ● = citrate-phosphate buffer (pH 4.0–5.0), △ and ▲ = phosphate buffer (pH 5.0–8.0), and □ and ■ = borate buffer (pH 8.0–9.0). Closed symbols represent ascorbic acid oxidase I and open symbols represent ascorbic acid oxidase II.

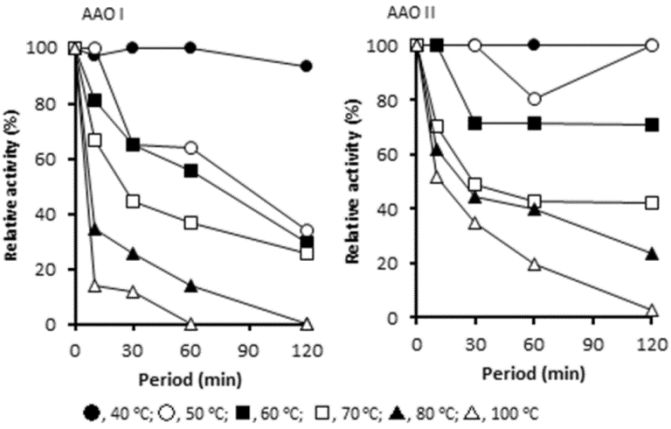

Fig. 7. Heat tolerance of ascorbic acid oxidase. Purified enzyme was heated at the temperature indicated for 30 min prior to rapid cooling and subsequent assay. AAOI and AAOII = ascorbic acid oxidase I and II, respectively.

TABLE II
Inhibition and Activation of Ascorbic Acid Oxidase[a]

	AAO I	AAO II
NaCl	100.3	87.4
KCl	91.3	91.6
Iodoacetate	15	0
$CuSO_4$	50.2	0
$MnSO_4$	112.2	109.1
$CoCl_2$	50.9	49.7
$HgCl_2$	0	0
$ZnSO_4$	107	121.4
CaCl2	18.6	58.9
$FeCl_2$	66.4	0
$MgSO_4$	27.4	9.3
BSF	18.8	67.2
NBS	6.9	41.7
EDTA	11.5	3.3
EGTA	16.2	0.3
Azide	43.5	66.2
Non	100	100

[a] Values show percent activity compared with control with no addition. All inhibitors were added at a final concentration of 1 m*M*. NBS, N-bromosuccinimide; BSF, benzenesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid.

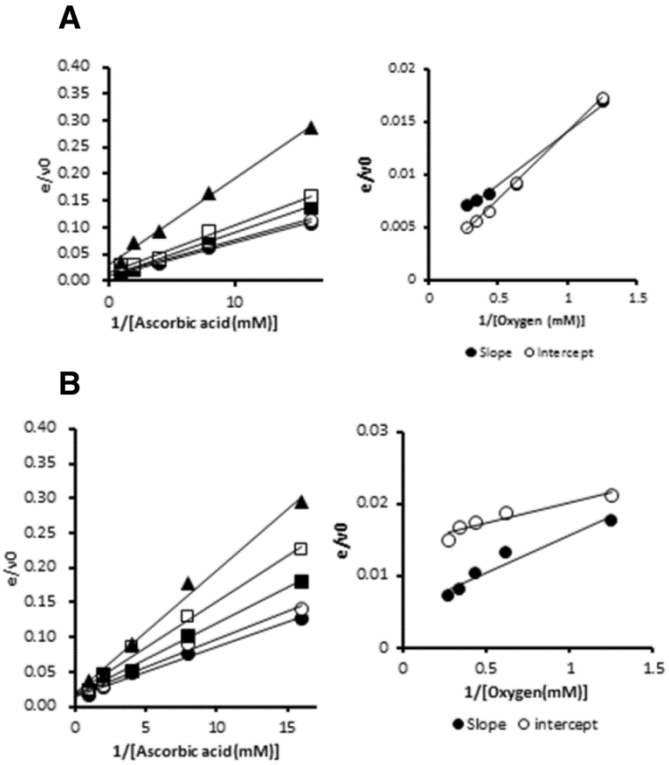

Fig. 8. Kinetic analysis of **A,** ascorbic acid oxidase I and **B,** ascorbic acid oxidase II. In each case, the left-hand plot depicts the relationship between activity and ascorbic acid concentration at a series of fixed oxygen concentrations whereas the right-hand plot gives the relationship between the slopes and the intercepts of the primary plots and oxygen concentration. Interpretation is as described in the footnote to Table III. Symbols on left graphs represent oxygen concentration: ● = 4 m*M*, ○ = 3.3 m*M*, ■ = 2.5 m*M*, □ = 1.7 m*M*, ▲ = 0.8 m*M*.

TABLE III
Kinetic Parameters of Ascorbic Acid Oxidase (AAO)[a]

Peak	Φ0 (s)	Φ1 (m*M* sec)	Φ2 (m*M* sec)	Φ12 (mM^2 sec)	1/Φ0 (s^{-1})	Φ1/ Φ0 (m*M*)	Φ2/Φ0 (m*M*)
AAOI	0.0012	0.0039	0.0129	0.0102	833	3.25	10.8
AAOII	0.0144	0.0051	0.0056	0.0105	69	0.35	0.39

[a] The Φ parameters are calculated from the secondary plots that are developed as described in the Materials and Methods section. Φ0 is the intercept on the ordinate of the secondary plot of ordinate intercepts of the primary plot against the reciprocal of the second substrate concentration. Φ2 is the slope of this line. Φ1 is the ordinate intercept of the plot of primary plot slopes against the reciprocal of the second substrate concentration. Φ12 is the slope of this line. 1/Φ0 represents the true maximum velocity (V_{max}). Φ1/Φ0 equals the K_m for the primary substrate. Φ2/Φ0 is the K_m for the secondary substrate. Ascorbic acid is the primary substrate and oxygen is the secondary substrate.

TABLE IV
Impact of Ascorbic Acid Additions in Mashing[a]

Time (min)	pH	Specific Gravity	Polyphenol (mg/L)	Thiols (A_{430})	Color
Plus ascorbic acid					
0	5.145 ± 0.015	1.0267 ± 0.0038	177 ± 3	0.315 ± 0.032	1.97 ± 0.18
10	5.28 ± 0.01	1.082 ± 0.0014	294 ± 2	0.483 ± 0.009	4.9 ± 0.43
20	5.335 ± 0.005	1.0852 ± 0.0015	321 ± 3	0.476 ± 0.025	6.66 ± 2.63
40	5.335 ± 0.015	1.0978 ± 0.0005	347 ± 14	0.478 ± 0.004	7.47 ± 1.25
60	5.385 ± 0.015	1.1061 ± 0.0011	384 ± 1	0.471 ± 0.048	9.5
Control					
0	5.58 ± 0	1.055 ± 0.006	177 ± 2	0.071 ± 0.003	5.42 ± 0.04
10	5.575 ± 0.005	1.081 ± 0.012	189 ± 2	0.06 ± 0.023	8.31 ± 0.83
20	5.5 ± 0.006	1.087 ± 0.009	198 ± 1	0.07 ± 0.007	7.44 ± 0.51
40	5.49 ± 0.02	1.093 ± 0.003	212 ± 3	0.059 ± 0.012	9.45 ± 0.37
60	5.485 ± 0.005	1.103 ± 0.002	236 ± 2	0.054 ± 0.009	9.68 ± 0.81

[a] A_{430} = absorbance at 430 nm; ± indicates mash-to-mash variation, with mashes being performed in duplicate.

mash decreased. The addition of AA led to substantially higher levels of polyphenol and thiols being measurable in the wort, this being consistent with the AA functioning as a substrate for AAO in consuming oxygen that would otherwise be used to oxidize polyphenols and thiols. There is generally also a lower color observed in the trial mashes (with the exception of the 60-min reading, which featured perhaps a spuriously high value). Again, this would be consistent with less polyphenol oxidation in the trial mashes.

DISCUSSION

Two AAO enzymes have been isolated from barley grain that are very different from any AAO previously reported (Table I). In the first instance, both are of much lower molecular size than previously reported activities. We find a weakly cationic enzyme of molecular weight in the region of 25,000 (AAOI) and a strongly cationic enzyme with an extremely low molecular weight of <10,000 (AAO II). The latter represents one of the smallest enzymes ever reported and would classify as a microenzyme (15,17).

Unsurprisingly, this enzyme is extremely thermotolerant but AAOI is also relatively heat resistant. Accordingly, there should be ample AAO activity in a mash at 65–70°C. Furthermore, both enzymes clearly are capable of operating at mashing pHs. In fact, it might be supposed that the major factor impacting the availability of this enzyme in a mash is the amount that is present in malt per se. The enzyme declines in level as germination is prolonged.

AAOII has much greater affinity (lower K_m) for both substrates than has AAO1, although the latter displays a higher V_{max} value. The K_m value for AA displayed by AAOII is comparable with that reported for AAO from other organisms (Table I). Few other papers report a K_m for oxygen but the value we have measured for AAOII is comparable with that from *Acremonium* spp. (11).

In view of the very high affinity of AAOII for AA and oxygen, coupled with its thermotolerance, we supposed that it ought to be capable of preferentially scavenging oxygen that would otherwise be expected to react enzymically or nonenzymically with other materials in a mash. As summarized by Stephenson et al (22), there are diverse potential events consequent to oxygen ingress in a mash, including possibilities for oxidation of unsaturated fatty acids, cross-linking of thiol-rich proteins, and oxidation of polyphenols with the production of color. It was our hypothesis, then, that the addition of AA to mashes would lead to a diminution in such effects. If, indeed, there was less oxidation of thiol groups, we would anticipate increased measureable levels of such groups in mashes containing AA. Similarly, we would expect an increased level of polyphenol surviving into wort and a decrease in the amount of color produced. Table IV shows that these expectations were realized. We are presently investigating whether AA added at this stage has any material impact on the flavor stability of beer.

LITERATURE CITED

1. American Society of Brewing Chemists. *Methods of Analysis*, St. Paul, MN, 2012.
2. Bamforth, C. W., and Martin, H. L. The development of β-glucan solubilase during barley germination. *J. Inst. Brew.* 87:81-84, 1981.
3. Bezerra Carvalho, L., Lima, C. J., and Medeiros, P. H. Ascorbate oxidase from *Cucurbita maxima*. *Phytochemistry* 20:2423-2424, 1981.
4. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254, 1976.
5. Dalziel, K. Initial state velocities in the evaluation of enzyme-coenzyme-substrate reaction mechanisms. *Acta Chem. Scand.* 11:1706-1723, 1957.
6. De Tullio, M. C., Ciraci, S., Liso, R., and Arrigoni, O. Ascorbic acid oxidase is dynamically regulated by light and oxygen. A tool for oxygen management in plants? *J. Plant Physiol.* 164:39-46, 2007.
7. De Tullio, M. C., Liso, R., and Arrigoni, O. 2004. Ascorbic acid oxidase: an enzyme in search of a role. *Biol. Plant* 48:161-166, 2004.
8. Every, D. Purification and characterization of ascorbate oxidase from flour and immature wheat kernels *J. Cereal Sci.* 30:245-254, 1999.
9. Honda, S. J. Ascorbic acid in barley roots. *Plant Physiol.* 30:174-181, 1955.

10. Hoy, J. L., Macauley, B. J., and Fincher, G. B. Cellulases of plant and microbial origin in germinating barley. *J. Inst. Brew.* 87:77-80, 1981.
11. Itoh, H., Hirota, A., Hirayama, K., Shin, T., and Murao, S. Properties of ascorbate oxidase produced by *Acremonium* sp. HI-25. *Biosci. Biotechnol. Biochem.* 59:1052-1056, 1995.
12. Kanauchi, M., and Bamforth, C. W. Ascorbate peroxidase in malted barley. *J. Am. Soc. Brew. Chem.* 71:97-102, 2013.
13. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-687, 1970.
14. Manchenko, G. P. *Handbook of Detection of Enzymes on Electrophoretic Gels*. CRC Press, Boca Raton, FL, 1994.
15. Mattey, M., Simoes, D., Brown, A., and Fan, X. Enzymes with a low molecular weight. *Acta Chim. Slov.* 45:45-57, 1998.
16. Mertz, D. Distribution and cellular localization of ascorbic acid oxidase in the maize root tip. *Am. J. Bot.* 48:405-413, 1961.
17. Monti, D., and Riva, S. Natural and artificial microenzymes: is it possible to have small and efficient biocatalysts? *Biocat. Biotrans.* 19:251-266, 2001.
18. Muller, R. J. Use of 5,5′-dithiobis (2-nitrobenzoic acid) as a measure of oxidation during mashing. *J. Am. Soc. Brew. Chem.* 53:53-58, 1995.
19. Oberbacher, M. F., and Vines, H. M. Spectrophotometric assay of ascorbic acid oxidase. *Nature* 197:1203-1204, 1963.
20. Porto, T. S., Porto, C. S., Cavalcanti, M. T., Filho, J. L., Perego, P., Porto, A. L., Converti, A., and Pessoa, A. Kinetic and thermodynamic investigation on ascorbate oxidase activity and stability of a *Cucurbita maxima* extract. *Biotechnol. Prog.* 22:1637-1642, 2006.
21. Schägger, H., and von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem.* 166:368-79, 1987.
22. Stephenson, W. H., Biawa, J.-P., Miracle, R. E., and Bamforth, C. W. Laboratory-scale studies of the impact of oxygen on mashing, *J. Inst. Brew.*, 109:273-283, 2003.
23. Szent-Györgyi, A. On the function of hexuronic acid in the respiration of the cabbage leaf. *J. Biol. Chem.* 90:385-393, 1931.
24. Tamas, L., Bocova, B., Huttova, J., Mistrik, I., and Olle, M. Cadmium-induced inhibition of apoplastic ascorbate oxidase in barley roots. *Plant Growth Regul.* 48:41-49, 2006.
25. Zelinová, V., Halušková, L., Mistrík, I., and Tamás, L. Abiotic stress-induced inhibition of root growth and ascorbic acid oxidase activity in barley root tip is associated with enhanced generation of hydrogen peroxide. *Plant Soil* 349:281-289, 2011.