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1Ascorbic acid oxidase in barley and malt and its possible role during mashing¹

2

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15 1. Preliminary reports of aspects of this work were present at the World Brewing Congress,

16 Portland OR, July/August 2012 and to the ASBC, Tucson, AZ, May, 2013

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19

20ABSTRACT

21

22 Ascorbic acid oxidase (AAO) develops in the embryo tissues of barley during steeping and initial
23stages of germination. Two AAO enzymes have been identified. One of them is of remarkably low
24molecular weight ($< 10,000$). Both are very heat tolerant and capable of acting over a broad pH range.
25Both enzymes would be expected to function during conversion temperatures of mashing. Indeed,
26addition of ascorbic acid to mashes results in the survival of higher levels of polyphenol and thiols into
27wort and a reduced color in that wort, commensurate with AAO preferentially consuming oxygen which
28is thus less readily available for other reactions in mashes including thiol oxidation and polyphenol
29oxidation.

30Key words: Ascorbic acid, embryo, heat resistant, mashing, molecular weight, oxidase

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39 Ascorbic acid oxidase (AAO; EC 1.10.3.3) has been known since 1931 when first identified (as
40 "hexoxidase") in cabbage leaf (23). Since then the enzyme has been widely reported in various plant and
41 fungal tissues (Table I), but as yet its precise function is under debate (7). It catalyzes the reaction

42

44

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

51

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

59

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

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64

65 **EXPERIMENTAL**

66

67 **Preparation of Malt**

68

69 Barley (100g, Metcalf, harvested in Canada, 2008) was germinated according to Hoy et al (10).
70 After treating the barley with 1% Sodium hypochlorite solution, it was washed with sterile water. Then
71 the barley was steeped in water at 16°C for 8 h, before draining and allowing to “air rest” at 16°C for 16
72 h. The barley was re-steeped in water at 16°C for 24 h prior to germinating at 16°C for 6 days. The green
73 malt was dried by lyophilization (Eyela FDU-2100, Tokyo Rikakikai Co. Ltd., Tokyo, Japan).

74

75 **Preparation of extracts**

76

77 Barley or malt was ground in a blender (AS ONE Model 7011HS cat#. 5340801) and extracted by
78 stirring on ice in 2.5 volumes of 2mM EDTA in phosphate buffer (50mM, pH 7.0) for 3 h at 4°C. The slurry

79 was then strained through cheesecloth and the resulting liquid was centrifuged at 10,000 x g. The
80 resulting supernatant was referred to as "crude extract".

81

82 Endosperm slices

83

84 Slices (2mm) were cut from sterile, dehusked barley at a distance of 2 mm behind the scutellum
85 (2). The slices were incubated at room temperature in sterile Petri-dishes (9 cm) containing gibberellic
86 acid (10^{-5} M; Sigma-Aldrich, cat#. 7645) sterilized by filter (Minisart SPR15). After incubation, 5 mL of
87 50mM phosphate Buffer (pH 7.0) was added and the tissues ground with a pestle and mortar. The
88 homogenate was kept for 1 h at room temperature before removal of particulate material by
89 centrifugation at 10,000 x g.

90

91 AAO assay

92

93 AAO activity was assayed on the basis of measuring the oxidation of ascorbate by the decrease
94 in absorbance at 265 nm ($\epsilon = 14 \text{ mM} \cdot \text{cm}^{-1}$) at 25°C (19). The reaction mixture contained 50mM potassium
95 phosphate buffer (pH 7.0), 0.5mM ascorbate, and enzyme solution. One unit of enzyme catalyzes the
96 oxidation of 1mM ascorbic acid per min.

97

98 AA peroxidase assay

99

100 AA peroxidase was assayed as described previously (12).

101

102 **Optimum pH determination**

103

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

106

107 **Purification of AAO**

108

109 Malt (100g), milled as described earlier, was extracted for 3h at 4°C with three volumes of 50mM
110 citrate-phosphate buffer pH7.0 containing 2mM EDTA and then centrifuged at 10,000 x g for 10min. The
111 enzyme extraction was applied to a column (25 mm × 300 mm) of Macro-Prep CM support (Bio-Rad CA
112 USA; http://www.bio-rad.com/LifeScience/pdf/Bulletin_9292.pdf). The protein was eluted using a 0–1 M
113 linear gradient of sodium chloride flowing at 1.5 mL min⁻¹. Fractions containing AAO were collected and
114 re-precipitated using 80% saturation of ammonium sulfate. The precipitate was re-dissolved in 2mL of
115 50mM citrate-phosphate pH 7.0, and then applied to the size-exclusion column (10 mm × 350 mm, P-100
116 gel, Bio-Rad CA USA). The eluant was citrate-phosphate, 50mM, pH 7.0 flowing at 2 mL min⁻¹.

117

118 **SDS-polyacrylamide gel electrophoresis**

11

12

119

120 The samples were separated on a 12.5% uniform gel (e-PAGEL, Cat# E-T12.5L, ATTO, Tokyo
121Japan) or a 15% uniform gel (e-PAGEL, Cat# E-T15S, ATTO, Tokyo Japan). Electrophoresis was carried out
122as described elsewhere (13, 21) with the following modifications: the samples (0.01 ml) were added to
1230.01 ml of sample buffer and then heated at 100°C for 3 min. Samples were added at 10 µl per well. The
124gels were run at 20 mA at a gel thickness of 1.5 mm. Molecular weight standards were from Bio-Rad
125Laboratories, Inc. The gel was stained with 0.25% Coomassie Brilliant Blue R-250.

126

127**Protein concentration determination**

128

129 Protein concentration was determined using the method of Bradford (4). The standard curve was
130produced using bovine serum albumin (Cat. # 05482: Sigma-Aldrich).

131

132

133**Location of AAO in grain using stains**

134

135 Barley or malt (1.0g) was steeped in 1% ascorbic acid solution in phosphate buffer (50mM, pH
1367.0) at 30°C for 1~4h. After reaction, residual ascorbic acid was detected by 1mL of 2,6-
137dichlorophenolindophenol solution (2.5mg in 100mL of deionized water) as described elsewhere (14).

138

139

140Heat stability experiment

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142 The enzyme solution was heated at 40–100°C for 30 min at pH 6.0 and cooled in ice prior to
143assay.

144

145

146Determination of kinetic parameters

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148 Two substrate kinetic analysis was conducted according to Dalziel (5) using a system as shown in
149Fig 1. Substrate solutions (1mL, 40°C) containing 0.0625mM, 0.125mM, 0.25mM, 0.5mM or 1.0mM
150ascorbic acid were introduced into the vial containing an electrode for measuring dissolved oxygen
151(TOKO chemical laboratory Co.Ltd, Tokyo, Japan) and sealed with a rubber seal. A vacuum was drawn in
152the vial and successively nitrogen gas (99.9%, Tomoe Shokai Co.), oxygen gas (99%, Tomoe Shokai Co.) or
153nitrogen-oxygen mixed gases (20-80% oxygen in nitrogen gas) was flushed through the vial. The
154operation was repeated three times. Enzyme solution was added by a micro-syringe through the seal.
155Decrease in A_{265} of the solutions was measured using a Nano-Drop 2000 (Thermo Fisher Scientific,
156Waltham MA).

157

158

159Impact of ascorbic acid additions in mashing

160

161 Pale Malt (2-row. Great Western Malting, Vancouver, WA) was milled in a Miag mill (MIAG
162Braunschweig, Dresden, Germany) on the coarse setting (0.7mm). Mashings at 65°C were performed in a
163bath (Canongate CM3, Canongate Technology Ltd, Edinburgh, UK) with 50g of milled malt, 150mg of
164ascorbic acid and 150mL of deionized water. The mashings designated for 0 minutes were filtered
165immediately upon mixing and subsequent mashings were removed at 10, 20, 40, and 60 minutes. Once
166removed, mashings were filtered through Whatman 2555 ½, 320 mm, cone filters into an ice bath. Wort
167samples were cooled to ~4°C and analyzed as soon as possible. pH was measured using an Orion
168Research expandable ionAnalyzer EA 920 after manual inversion. After cone filtration, between 5 and 10
169mL samples of wort were passed through glass fiber filters. The resulting sample was run through an
170Anton Paar DMA 4100m density meter to determine specific gravity. Color was determined by taking the
171absorbance at 430nm of the remaining sample in a plastic cuvette (1). Total polyphenols were measured
172in accordance with the ASBC method (1). Free thiols were determined using the method of Muller (18).

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RESULTS

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181Enzyme properties

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183 AAO is not present in ungerminated barley but starts to be synthesized immediately upon
184steeping (Fig 2). It reaches a maximum level of activity early in germination, thereafter decreasing to a
185low but finite level at the end of germination. By contrast, ascorbate peroxidase is synthesized rather
186later

187

188 AAO is primarily located in the embryo of dissected sprouted grain with successively less enzyme
189in the proximal and distal endosperm (data not shown).

190

191 Fractionation of crude extracts of malt by cation exchange chromatography revealed 2 peaks of
192AAO activity (Fig 3), which are designated AAOI and AAOII. Further chromatography of these peaks on
193Bio-Gel P100 indicated that AAOI was of higher molecular size than is AAOII (Fig 4a, b) and this was
194confirmed by polyacrylamide gel electrophoresis (Fig 5). Molecular weight estimates for the two
195enzymes are approximately 25-27,000 and 6-9,000 respectively.

196

197 Both enzymes had a pH optimum of approximately 7, but AAOI has a broader activity range (Fig
1986). Both enzymes would be expected to display significant activity at mashing pHs and in beer.

199

200 Both enzymes are relatively heat tolerant (Fig 7). Of the two, the very low molecular weight
201AAOII is really rather phenomenally thermotolerant, with some 20% of the activity surviving a 1-hour
202boil.

203

204 The enzyme may display slight activation by manganese and zinc, however it is inhibited by
205copper, despite AAO in most plants generally being described as an enzyme rich in copper (Table II).
206Strong inhibition by EDTA and EGTA would be consistent with the need for a metal ion in the action of
207the enzymes, though iron and magnesium (and mercury) are potent inhibitors, especially of AAOII.
208Inhibition by azide suggests the presence of a functional heme group in the enzyme. Inhibition by
209iodoacetate indicates a functional presence of thiol groups. Inhibition by N-bromosuccinimide (NBS)
210suggests a role for tryptophan and inhibition by benzenesulfonyl fluoride (BSF) would be consistent with
211a role for a serine group.

212

213 Two substrate kinetic analysis (Figs 8a, b; Table III) revealed that AAOI is capable of operating
214faster than AAOII (higher V_{max}). However AAOII has a much greater affinity for both substrates (lower K_m
215values).

216

217Mashing studies

218

219 Mashers were performed at 65°C either in the presence or absence of 5.7mM ascorbic acid. This
220value is well in excess of the K_m value for AAOII, which would thus be expecting to operate at maximum

221rate at the start of mashing (Table IV). The presence of ascorbic acid had little impact on the specific
222gravity of recovered worts. Unsurprisingly it lowered the pH of the mash, although this rose
223progressively through mashing whereas the pH of the control mash decreased. The addition of ascorbic
224acid led to substantially higher levels of polyphenol and thiols being measurable in the wort, this being
225consistent with the ascorbic acid functioning as a substrate for AAO in consuming oxygen that would
226otherwise be used to oxidize polyphenols and thiols. There is generally also a lower color observed in the
227trial mashes (with the exception of the 60 minute reading which featured perhaps a spuriously high
228value). Again this would be consistent with less polyphenol oxidation in the trial mashes.

229

230

231

DISCUSSION

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233 Two AAO enzymes have been isolated from barley grain that are very different from any AAO
234previously reported (c.f. Table I). In the first instance, both are of much lower molecular size than
235previously reported activities. We find a weakly cationic enzyme of molecular weight in the region of
23625,000 (AAOI) and a strongly cationic enzyme with an extremely low molecular weight of less than
23710,000 (AAO II). The latter represents one of the smallest enzymes ever reported and would classify as a
238microenzyme (15, 17).

239

240 Unsurprisingly, this enzyme is extremely thermotolerant, but AAOI is also relatively heat
241resistant. Accordingly there should be ample AAO activity in a mash at 65-70°C. Furthermore both
242enzymes clearly are capable of operating at mashing pHs. In fact it might be supposed that the major

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316growth and ascorbic acid oxidase activity in barley root tip is associated with enhanced generation of
317hydrogen peroxide. Plant Soil, 349:281–289, 2011

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325Table I. Comparison of ascorbic acid oxidases from different organisms

Organism	Molecular weight	pH optimum	Km ascorbic acid (mM)	Km oxygen (mM)	Heat tolerance	Inhibitors	Activators	References
Barley						cadmium	cadmium	24
Wheat	139,000 (Dimer)	6.2	0.3		Stable at 40°C for 30 minutes			8
Maize		5.8						6
Squash	150,000 (dimer)	5.5 (max at 7.0); 6.0	0.2		Half life of 21 minutes at 70°C; Destroyed in <1 minute at 100°C	azide, thiourea		3, 20
<i>Acremonium</i>	80,000	4.0	0.29	0.47	Survives 30 min at 60°C	azide, cyanide, Fe ²⁺ , H ₂ S	copper	11

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341Table II Inhibition and activation of Ascorbic acid oxidase

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	AAO I	AAO II		AAO I	AAO II
NaCl	100.3	87.4	FeCl ₂	66.4	0.0
KCl	91.3	91.6	MgSO ₄	27.4	9.3
Iodoacetate	15.0	0.0	BSF	18.8	67.2
CuSO ₄	50.2	0.0	NBS	6.9	41.7
MnSO ₄	112.2	109.1	EDTA	11.5	3.3
CoCl ₂	50.9	49.7	EGTA	16.2	0.3
HgCl ₂	0.0	0.0	Azide	43.5	66.2
ZnSO ₄	107.0	121.4	Non	100.0	100.0
CaCl ₂	18.6	58.9			

343

344Values show % activity compared to control with no addition. All inhibitors were added at a final
345concentration of 1mM.

346

347NBS, N-bromosuccinimide; BSF, benzenesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; EGTA,
348ethyleneglycoltetraacetic acid

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361 Table III Kinetic Parameters of Ascorbic Acid Oxidase ^a

	Φ_0 (s)	Φ_1 (mM.s)	Φ_2 (mM.s)	Φ_{12} (mM ² .s)	$1/\Phi_0$ (s ⁻¹)	Φ_1/Φ_0 (mM)	Φ_2/Φ_0 (mM)
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

362

363

364

365^a The φ parameters are calculated from the secondary plots that are developed as described in the
 366 Materials and Methods section. φ_0 is the intercept on the ordinate of the secondary plot of ordinate
 367 intercepts of the primary plot against the reciprocal of the second substrate concentration. φ_2 is the
 368 slope of this line. φ_1 is the ordinate intercept of the plot of primary plot slopes against the reciprocal of
 369 the second substrate concentration. φ_{12} is the slope of this line. $1/\varphi_0$ represents the true maximum
 370 velocity (V_{max}). φ_1/φ_0 equals the K_m for the primary substrate. φ_2/φ_0 is the K_m for the secondary
 371 substrate. Ascorbic acid is the primary substrate and oxygen is the secondary substrate.

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386Table IV The impact of ascorbic acid additions in mashing

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388 (a) Plus ascorbic acid

Time (min)	pH	Specific Gravity	Polyph enol (mg/L)	Thiols (A ₄₃₀)	Color
		1.0267±0.0038		0.315±0.032	1.97±0.18
0	5.145±0.015	1.082±0.0014	177±3	0.483±0.009	4.9±0.43
10	5.28±0.01	1.0852±0.0015	294±2	0.476±0.025	6.66±2.63
20	5.335±0.005	1.0978±0.0005	321±3	0.478±0.004	7.47±1.25
40	5.335±0.015	1.1061±0.0011	347±1	0.471±0.048	9.5
60	5.385±0.015		384±1		

(b) Control

		1.055±0.006		0.071±0.003	5.42±0.04
0	5.58±0	1.081±0.0012	177±2	0.06±0.023	8.31±0.83
10	5.575±0.005	1.087±0.0009	189±2	0.07±0.007	7.44±0.51
20	5.5±0.006	1.093±0.0003	198±1	0.059±0.012	9.45±0.37
40	5.49±0.02	1.103±0.0002	212±3	0.054±0.009	9.68±0.81
60	5.485±0.005		236±2		

389

390

391± indicates mash to mash variation, with mashes being performed in duplicate

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396

397*Legends to Figures*

398

399Fig 1. Apparatus for determining kinetic parameters for Ascorbic Acid Oxidase

400Air is removed from the vial using vacuum with stopcock C open. To adjust oxygen concentration, a
401mixture of oxygen and nitrogen (e.g. 10mL of O₂ and 90mL of N₂) is transferred to the measuring cylinder
402filled with saturated NaCl solution via stopcock B. Upon closing B and opening stopcock A, the gas
403mixture is sucked vigorously to the vial from cylinder. The operation is repeated three times to achieve a
404stable oxygen content as measured using the dissolved oxygen meter. Enzyme is added to the substrate
405mixture by micro-syringe. After reaction, 10uL of the vial contents are transferred by micro-syringe for
406measurement of A₂₆₅ by Nanodrop 2000.

407

408

409Fig 2. Levels of Ascorbic Acid Oxidase and ascorbate peroxidase during the steeping and germination of
410barley.

411The zero time point represents the barley prior to steeping. Enzyme values are quoted as units per g
412malt.

413

414Fig 3. Ion exchange chromatography of Ascorbic Acid Oxidase.

415

416 Fig 4 Gel permeation chromatography of Ascorbic Acid Oxidase (a) Ascorbic Acid Oxidase I (b) Ascorbic
417 Acid Oxidase II.

418

419 Fig 5. Molecular weight determination for Ascorbic Acid Oxidase. **A**, SDS-Polyacrylamide gel
420 electrophoresis. The left hand illustration represents Ascorbic Acid Oxidase I, the right hand one
421 represents Ascorbic Acid Oxidase II. In each case the lane to the left shows molecular weight standards
422 and the right hand lane the purified enzyme. **B**, By gel permeation chromatography. The filled circles
423 indicate reference proteins. □, Ascorbic Acid Oxidase I; ◇, Ascorbic Acid Oxidase II

424

425 Fig 6. pH optimum for Ascorbic Acid Oxidase.

426 ○&●: citrate-phosphate buffer (pH 4.0–5.0), △&▲: phosphate buffer (pH 5.0–8.0), □&■: borate buffer
427 (pH 8.0–9.0). Closed symbols represent AAOI; open symbols represent AAOII.

428

429 Fig 7 Heat tolerance of Ascorbic Acid Oxidase. Purified enzyme was heated at the temperature indicated
430 for 30 minutes prior to rapid cooling and subsequent assay

431

432 Fig 8 Kinetic analysis of (a) Ascorbic Acid Oxidase I (b) Ascorbic Acid Oxidase II. In each case the left
433 hand plot depicts the relationship between activity and ascorbic acid concentration at a series of fixed
434 oxygen concentrations whereas the right hand plot gives the relationship between the slopes and the

435intercepts of the primary plots and oxygen concentration. Interpretation is as described in the footnote
436to Table III.

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Fig.2

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Fig 3

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Fig 4a

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Fig 4b

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Fig 5

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Fig 8a

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Fig 8b

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