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Authors

Kanauchi, Makoto
Simon, Kyle J
Bamforth, Charles W

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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*; **Kyle J. Simon and Charles W. Bamforth[2],** *Department of Food Science and Technology, University of California, Davis, CA 95616, USA*

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. E-mail: cwbamforth@ucdavis.edu; phone: +1-530-752-9476; fax: +1-530-752-4759

20ABSTRACT

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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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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

$$2\ \text{L-ascorbate} + O_2 \rightarrow 2\ \text{dehydroascorbate} + 2\ H_2O \qquad (1)$$

Reports of AAO in barley are limited. Honda studied the enzyme in barley roots and found it be associated with cell walls (9), in keeping with the findings of others (16). Tamas et al (24) studied the impact of cadmium on AAO in germinating barley seeds, with the agent causing substantial inhibition of rootlet growth. Cadmium inhibited two cationic AAO enzymes and also two anionic isozymes. A fifth AAO, this one cationic, was activated by cadmium. 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 (100g, Metcalf, harvested in Canada, 2008) was germinated according to Hoy et al (10). After treating the barley with 1% Sodium hypochlorite solution, it was washed with sterile water. Then the barley was steeped in water at 16°C for 8 h, before draining and allowing to “air rest” at 16°C for 16 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 malt was dried by lyophilization (Eyela FDU-2100, Tokyo Rikakikai Co. Ltd., Tokyo, Japan).

Preparation of extracts

Barley or malt was ground in a blender (AS ONE Model 7011HS cat#. 5340801) and extracted by 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

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

Endosperm slices

Slices (2mm) 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, cat#. 7645) sterilized by filter (Minisart SPR15). After incubation, 5 mL of 50mM phosphate Buffer (pH 7.0) was added and the tissues ground with a pestle and mortar. The homogenate was kept for 1 h at room temperature before removal of particulate material by centrifugation at 10,000 x *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 $mM.cm^{-1}$) at 25°C (19). The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM ascorbate, and enzyme solution. One unit of enzyme catalyzes the oxidation of 1mM ascorbic acid per min.

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 (100g), milled as described earlier, was extracted for 3h at 4°C with three volumes of 50mM citrate-phosphate buffer pH7.0 containing 2mM EDTA and then centrifuged at 10,000 x g for 10min.The enzyme extraction was applied to a column (25 mm × 300 mm) of Macro-Prep CM support (Bio-Rad CA USA; 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 re-precipitated using 80% saturation of ammonium sulfate. The precipitate was re-dissolved in 2mL of 50mM citrate-phosphate pH 7.0, and then applied to the size-exclusion column (10 mm × 350 mm, P-100 gel, Bio-Rad CA USA). The eluant was citrate-phosphate, 50mM, pH 7.0 flowing at 2 mL min^{-1}.

SDS-polyacrylamide gel electrophoresis

The samples were separated on a 12.5% uniform gel (e-PAGEL, Cat# E-T12.5L, ATTO, Tokyo Japan) or a 15% uniform gel (e-PAGEL, Cat# E-T15S,ATTO, Tokyo Japan). 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℃ 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 (Cat. # 05482: Sigma-Aldrich).

Location of AAO in grain using stains

Barley or malt (1.0g) was steeped in 1% ascorbic acid solution in phosphate buffer (50mM, pH 7.0) at 30℃ for 1~4h. After reaction, residual ascorbic acid was detected by 1mL of 2,6-dichlorophenolindophenol solution (2.5mg in 100mL of deionized water) as described elsewhere (14).

Heat stability experiment

The enzyme solution was heated at 40–100℃ 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 Fig 1. Substrate solutions (1mL, 40°C) containing 0.0625mM, 0.125mM, 0.25mM, 0.5mM or 1.0mM ascorbic acid were introduced into the vial containing an electrode for measuring dissolved oxygen (TOKO chemical laboratory Co.Ltd, Tokyo, Japan) and sealed with a rubber seal. A vacuum was drawn in the vial and successively nitrogen gas (99.9%, Tomoe Shokai Co.), oxygen gas (99%, Tomoe Shokai Co.) or nitrogen-oxygen mixed gases (20-80% oxygen in nitrogen gas) was flushed through the vial. The operation was repeated three times. Enzyme solution was added by a micro-syringe through the seal. Decrease in A_{265} of the solutions was measured using a Nano-Drop 2000 (Thermo Fisher Scientific, Waltham MA).

Impact of ascorbic acid additions in mashing

Pale Malt (2-row. Great Western Malting, Vancouver, WA) was milled in a Miag mill (MIAG Braunschweig, Dresden, Germany) on the coarse setting (0.7mm). Mashes at 65°C were performed in a bath (Canongate CM3, Canongate Technology Ltd, Edinburgh, UK) with 50g of milled malt, 150mg of ascorbic acid and 150mL of deionized water. The mashes designated for 0 minutes were filtered immediately upon mixing and subsequent mashes were removed at 10, 20, 40, and 60 minutes. Once removed, mashes were filtered through Whatman 2555 ½, 320 mm, cone filters into an ice bath. Wort samples were cooled to ~4**°**C and 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 430nm 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

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181**Enzyme 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

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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).

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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 AAO1 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.

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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.

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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-hour 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 (NBS) suggests a role for tryptophan and inhibition by benzenesulfonyl fluoride (BSF) would be consistent with a role for a serine group.

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

Mashing studies

Mashes were performed at 65^0C either in the presence or absence of 5.7mM ascorbic acid. This value is well in excess of the K_m value for AAOII, which would thus be expecting to operate at maximum

rate at the start of mashing (Table IV). The presence of ascorbic acid 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 mash decreased. The addition of ascorbic acid led to substantially higher levels of polyphenol and thiols being measurable in the wort, this being consistent with the ascorbic acid 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 minute 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 (c.f. 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 less than 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 ascorbic acid displayed by AAOII is comparable with that reported for AAO from other organisms (c.f. 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* (11).

In view of the very high affinity of AAOII for ascorbic acid 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 non-enzymically 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 ascorbic acid 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 –SH in mashes containing ascorbic acid. 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 ascorbic acid added at this stage has any material impact on the flavor stability of beer.

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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
Acremonium	80,000	4.0	0.29	0.47	Survives 30 min at 60°C	azide, cyanide, Fe^{2+}, H_2S	copper	11

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341Table II Inhibition and activation of Ascorbic acid oxidase

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	AAO I	AAO II
NaCl	100.3	87.4
KCl	91.3	91.6
Iodoacetate	15.0	0.0
$CuSO_4$	50.2	0.0
$MnSO_4$	112.2	109.1
$CoCl_2$	50.9	49.7
$HgCl_2$	0.0	0.0
$ZnSO_4$	107.0	121.4
CaCl2	18.6	58.9

	AAO I	AAO II
$FeCl_2$	66.4	0.0
$MgSO_4$	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	100.0	100.0

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

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

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Table III Kinetic Parameters of Ascorbic AcidOxidase [a]

	Φ0 (s)	Φ1 (mM.s)	Φ2 (mM.s)	Φ12 (mM^2.s)	1/ Φ0 (s^{-1})	Φ1/ Φ0 (mM)	Φ2/ Φ0 (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

[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.

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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	Polyphenol (mg/L)	Thiols (A_{430})	Color
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±0.014	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
	(b) 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

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

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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 O_2 and 90mL of N_2) 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 A_{265} by Nanodrop 2000.

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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

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

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

424

425Fig 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

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

431

432Fig 8 Kinetic analysis of (a) Ascorbic Acid Oxidase I (b) Ascorbic Acid Oxidase II. In each case the left
433hand plot depicts the relationship between activity and ascorbic acid concentration at a series of fixed
434oxygen 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.1

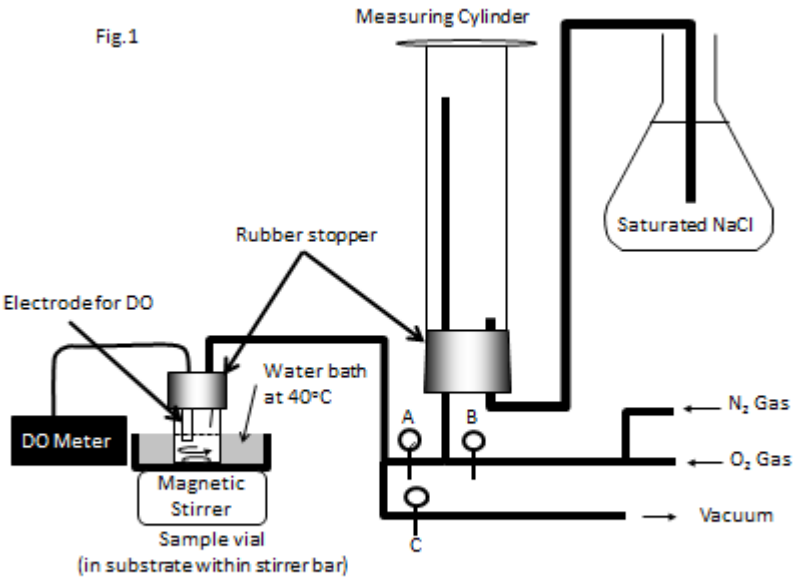

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Fig.2

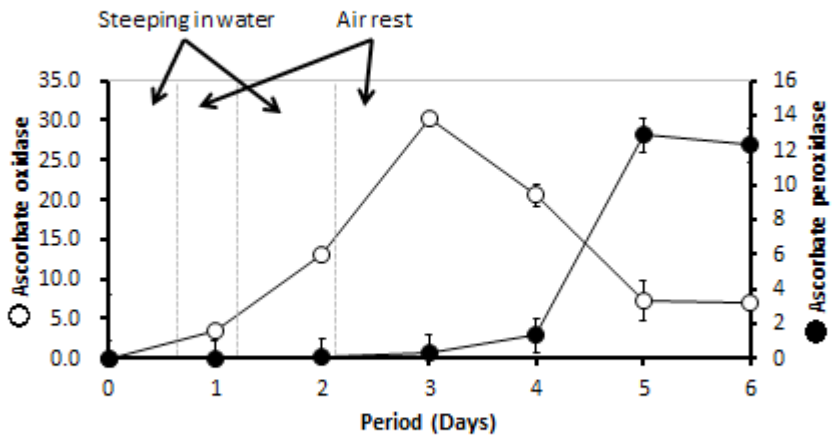

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Fig 3

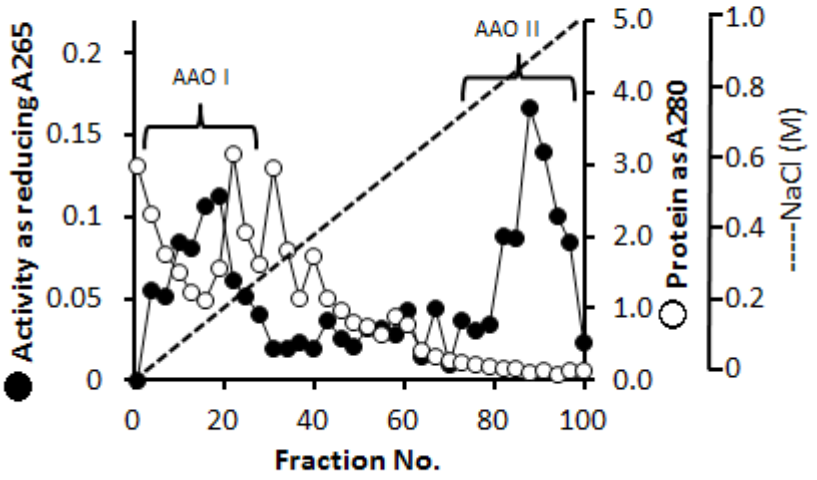

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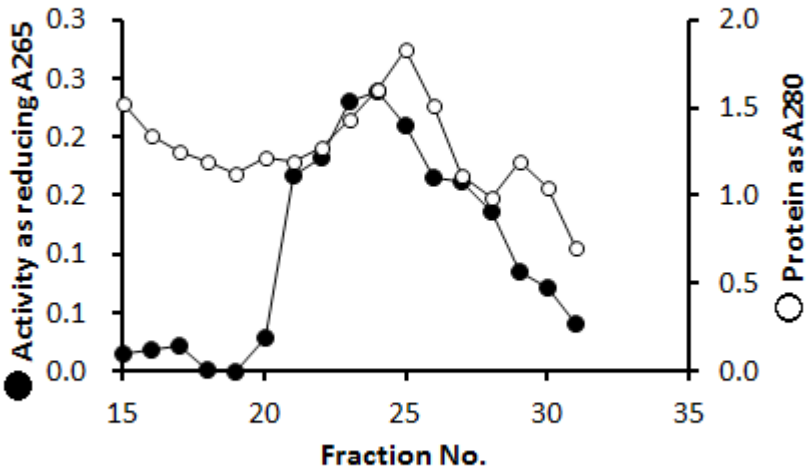
Fig 4a



447

Fig 4b

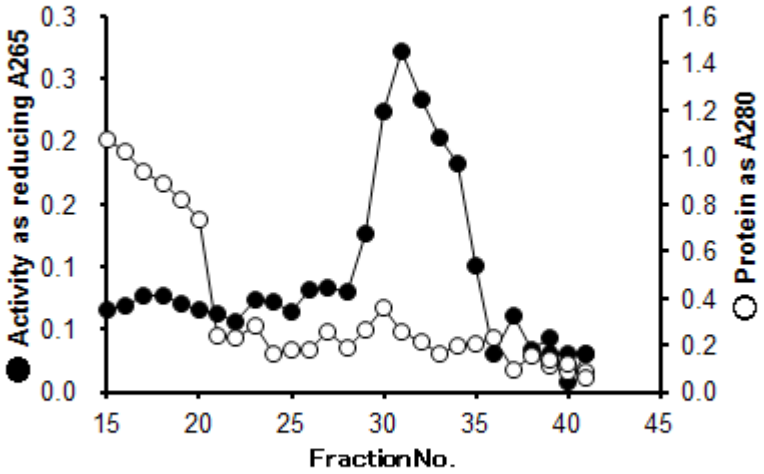


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Fig 5

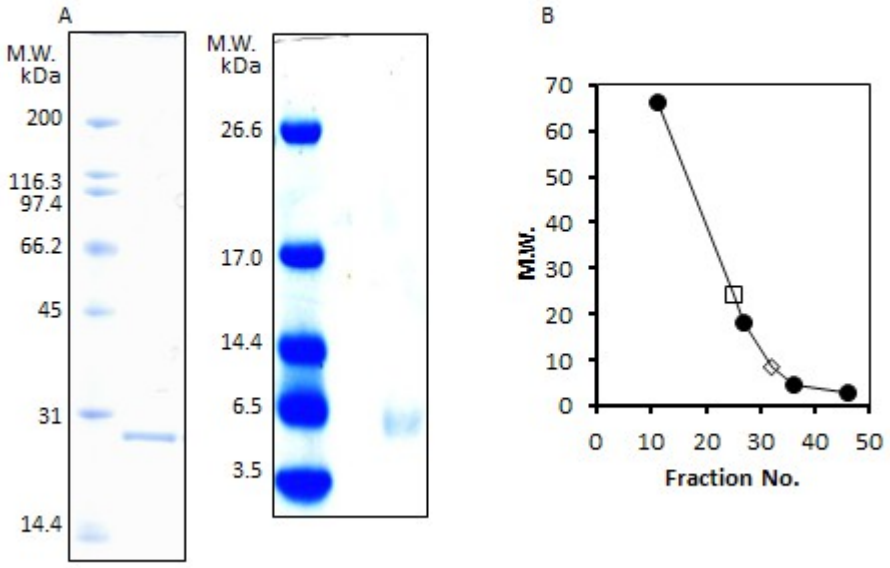

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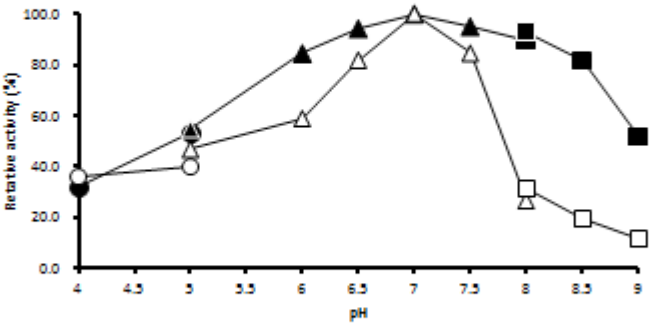
Fig.6



454

Fig 7

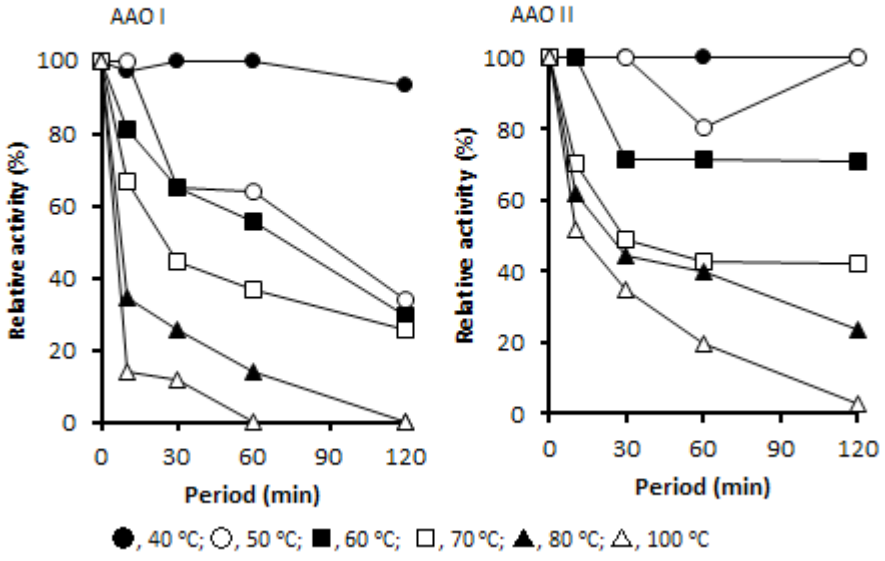


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Fig 8a

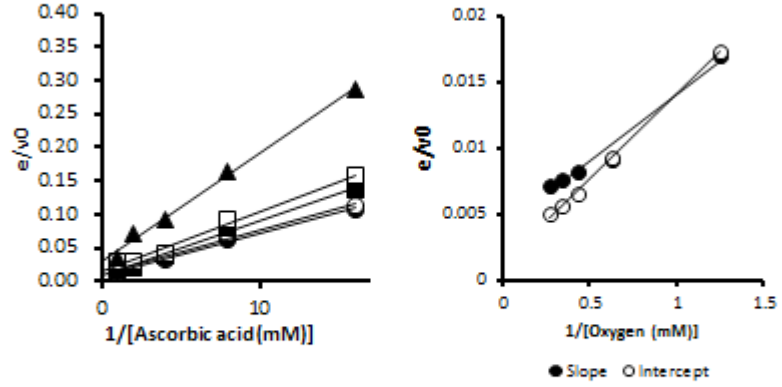

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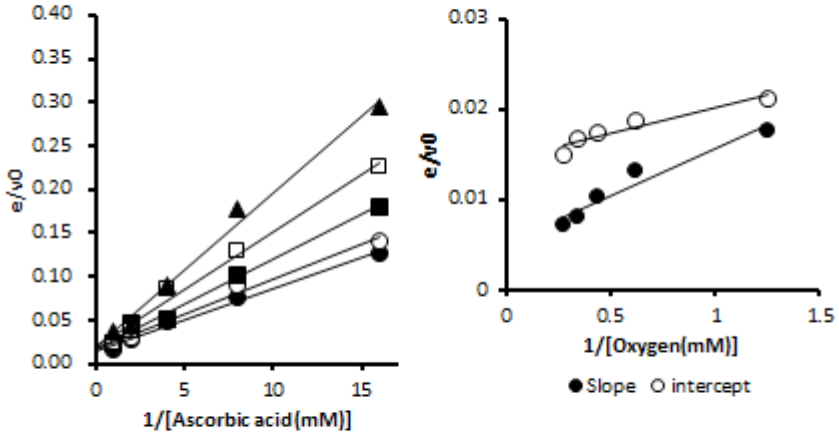
Fig 8b



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