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Oxidative Enzyme Effects in Malt for Brewing

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<http://dx.doi.org/10.5772/intechopen.69803>

Abstract

Malted barley is an important beer-brewing material that strongly affects brewing processes, the aroma, and the taste of beer. In addition to imparting a good aroma, malt not only generates substrates and enzymes, such as starches and some amylase, for alcohol production but also generates beer-quality-degrading substances and enzymes. Four oxidases are specifically addressed in this chapter. First, thiol oxidase in malt is described. The activity of thiol oxidase decreases during malt storage. Next, ascorbate peroxidase was investigated. It has been detected in the acrospires and aleurones of germinating barley. The enzyme has extremely high affinity for hydrogen peroxide. Also, ascorbic acid oxidase (AAO) was investigated. It is developed in the embryo tissues of barley during steeping and during the initial stages of germination. The addition of ascorbic acid to mash leads to the survival of higher levels of polyphenol and thiols into wort and a reduced color in that wort. Finally, oxalate oxidase in barley kernels is described. It is probably less important than other oxidases in scavenging oxygen from mashes, because the enzyme has low affinity for oxygen. Beer quality is expected to be improved by the regulation of oxidant enzymes, such as thiol oxidase or AAO, oxalate oxidase, or substrates, such as oxygen.

Keywords: malt, oxidation enzymes, thiol, ascorbic acid, hydrogen peroxide, oxalic acid

1. Introduction

1.1. Malt science

Malted barley is an important material used in producing beer. Maltose is made from starch by the enzyme developed during barley germination. Yeast ferments sugars to alcohol. Components and enzymes of malt are important for beer quality. This chapter presents an explanation of cell wall oxidase in malt and germinated barley.

Kernels of barley (*Hordeum vulgare*) are of two types: two-row and six-row. Two-row barley is cultivated in Australia, Canada, and Europe and six-row barley is cultivated in the USA. The barley grain covers a glume. The endosperm is protected by the husk, which is fractured by a mechanical shock during malting. Commercial barleys have the husk removed from the grain. The husk is largely insoluble, forming a filter bed for lautering in the brewing process. The pericarp is waxy and waterproof. The testa acts as a permeable membrane. Water readily penetrates into the embryo through the testa during steeping. The seed of the barley and the embryo and aleurone layer are living. After the kernel absorbs water, gibberellin is excreted as a phytohormone from the germ. Furthermore, the signal of gibberellin leads to the production of enzymes, such as amylase, and to the synthesis of β -glucanase to decompose starch. The reaction transitions from endosperm verging of the aleurone or germ to internal endosperm for decomposition of starch [1].

Barley for brewing must meet six necessary conditions [1]:

1. Grains are large, with uniform size and shape.
2. Barley has a thin husk.
3. Grains contain uniform protein concentration and rich starch.
4. Grains have vigorous germination evenly.
5. Germinated grains have a high level of enzyme activity.
6. Germinated grains (malt) can saccharify easily and can ferment quickly for wort.

1.2. History of beer

Ancient beer called 'SIKARU' was brewed from Emmer wheat in Mesopotamia in 3000 BC. In Egypt, bread made from germinated barley was dissolved with water and fermented in a vase. It is not modern beer. At about that time, 'GRUUT,' a herbal mixture of *Myrica gale*, mugwort, or others, was used for beer production in Medieval Europe. Hops have been used for beer-brewing since the twelfth to the fifteenth century. Modern beer, with its fresh aroma, bitterness, and rich foaming is developed from the use of hops. The purpose of using hops has been the subject of various theories. However, hops might be useful mainly in preventing the growth of lactic acid bacteria [1].

1.3. Consumption of beer

Beer is the most consumed alcoholic beverage in the world. Annual global consumption was 146 million kL in 2015. People in China, who consumed 43 million kL of beer in 2015, consume more beer than is consumed in any other country. The second leading country for consumption is the USA, with 22 million kL of beer consumed, followed in order by Brazil, Germany, and Mexico. These five countries collectively consume more than 60% of all the beer produced. Japan is ranked seventh, with 5 million kL of beer consumption (Kirin Co. Ltd.) [2].

Japan has a unique system of liquor tax laws. Beer is categorized legally according to the ratio of malt and other starchy materials [1]. Beer is defined legally in Japan as described below:

- (i) Beer is fermented from materials, such as malt, hops, and water.
- (ii) Beer is fermented malt, hops, water, and other legally allowed materials, such as barley, rice, sorghum, corn, potato, starch, and sugars. However, the mass of the materials must be less than 50% of the mass of malt.

Before the law changed in 1996, legally allowed materials were less than one-third of the malt quantity. Japanese brewery companies sold low-malt, low-tax, and low-cost beer. After changing the law in 2006, beer or malt beverages came to be classified into three categories: first, the ratio of malt among all starch materials is greater than 50%; second, the ratio of malt among all starch materials is 25–50%; and third, the ratio of malt among all starch materials is less than 25%. Low-malt, low-tax, and low-cost beer is known as ‘HAPPOUSHU,’ which is generally brewed from less than 25% malt among all starch materials [1].

2. Oxidative enzyme in malt

Generally, two types of oxidations occur in food: enzymic oxidation and nonenzymic oxidation. By enzymic oxidation, an apple changes color because of the effects of polyphenol oxidase. By nonenzymic oxidation, auto-oxidation of lipids occurs by the production of peroxide. Beer also has oxidants and two similar oxidation systems. Their oxidation types are important factors affecting beer quality. In this section, effects of oxidation enzymes in brewing processes are presented in Table 1 [3–7].

2.1. Thiol oxidase

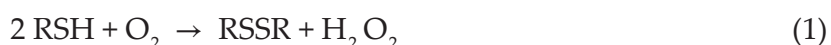
To avoid oxidation during germination, freshly kilned malt is typically not stored for more than 2–4 weeks. This storage inability presents problems, notably reduced rates of wort separation [8].

Flavor:
lipid oxidation; LOX or peroxidase [3]
Higher alcohol oxidation [3]
Forming haze:
Oxidation oxidized polyphenols tend to cross-link with protein [4]
Dark color:
Second source of coloring, oxidation of polyphenol or tannic material [4]
Difficult wort-run off:
Hordein protein-containing cysteine having sulphide bond. After Mash oxidizes during mashing, wort runs off a filter slowly, because of Hordein protein undergo oxidative polymerization as disulfide bond [5–7]

Table 1. Effects of oxidation enzymes on beer quality.

Freshly kilned malt is unsuitable for mashing [9]. Furthermore, saccharification also occurs less readily and the wort becomes turbid. It might also give rise to poor fermentation and haze in the beer. Therefore, storage for about three weeks before use is conducted as a precaution.

The oxidation of peptides having thiol-amino residue as cysteine produces gel proteins, which impede wort separation [7]. Reduced protein-containing thiol groups engenders acceleration of wort filtration [10]. Muller [11] reported the production of hydrogen peroxide in mashes attendant to the oxidation of thiol groups. Stephenson et al. [12] reported that some share of thiol oxidation occurring in mashes is enzyme-catalyzed. Malt extracts oxidized the cysteine to the equivalent dithiol cysteine. When malt extract was denatured by boiling, the interchange did not cause enzymic oxidation, such as thiol oxidase. The enzyme has Enzyme Commission number EC 1.8.3.2.



Bamforth et al. [13] reported the isolation of such an enzyme from malt and discussed some of its salient properties. Barley was germinated as described in an earlier report by Hoy et al. [14]. The green malt was dried at 60°C for 16 h. Thereafter, the malt was stored in incubators at 10, 20, or 30°C. Barley or germinated barley was ground in a coffee grinder. Then the crude extract was extracted by stirring on ice in buffer containing 0.25 M NaCl for 1 h. The slurry was then strained through cheesecloth. The resulting liquid was centrifuged at 13,000 rpm for 10 min. Denatured samples were the supernatants of crude extracts autoclaved using a laboratory autoclave switched to the “liquid” setting adjusted to sterilize for 15 min. Furthermore, thiol oxidation assay was conducted on extract of raw or malted barley that were incubated at 25°C in a potassium phosphate buffer (pH 7.5)-containing ethylenediaminetetraacetic acid (EDTA) together with cysteine or another reduced substrate (dithiothreitol, glutathione, or mercaptoethanol). Aliquots (0.15 mL) were removed at intervals for the determination of thiol using Ellman’s [15] reagent (5,5′-dithiobis (2-nitrobenzoic acid) [DTNB]) by measuring the light absorbance in a spectrophotometer at 412 nm. To purify thiol oxidase, malt milled as described earlier was extracted for 3 h at 4°C with borate buffer (pH 9.0)-containing 2 mM EDTA and 0.5% NaCl. After centrifugation of the extract, thiol oxidase was purified by ammonium sulfate precipitation, a column of DEAE gel (Macro-Prep DEAE, 25 mm × 300 mm, Bio-Rad, Hercules, CA, USA), and a size-exclusion column (10 mm × 350 mm, P-100 gel, Bio-Rad).

The enzyme had a molecular weight of 35,700 Da. Thiol oxidase had a pH optimum of approximately pH 8.0. During mashing, the pH of the enzymic activity was low. The pH in mash, such as pH 5.0 was too low for the thiol oxidase reaction. Storage at 20°C led to the virtual elimination of activity after 4 weeks, but at 30°C, the activity was lost completely after 2 weeks. The enzyme decreased the rate of filtration of gel proteins because it promotes the cross-linking of these molecules.

The extent of the reduction of filtration was also greatest under alkaline conditions. However, it did not agree with the optimum pH as determined in the enzyme assay. The very low activity of enzyme extract at pH 5.0 is worth exploring a little more. Bearing in mind this possibility, the presumed enzyme catalyzing the oxidation of thiols was observed to be resistant to more

modest degrees of heat. This enzyme has heat tolerance. The residual appearance enzyme activity at 100°C was approximately 50% of that of the nonheat enzyme. High thermal tolerance has been claimed for similar enzymes in other organisms [16–18].

In speculation of enzyme specificity, the enzyme was extremely active with cysteine as the substrate. It had much less activity with reduced glutathione (a tripeptide of glutamate, cysteine, and glycine), mercaptoethanol, and dithiothreitol (thiol compound often used in biochemical systems). Malt contains a heat-stable thiol oxidase. It is far from its optimum pH (pH 8.0) when at pH 5, which is its general mashing pH. It can be predicted that lowering the mashing pH would lessen the extent of its action. The activity of thiol oxidase lessens during malt storage, which suggests that this is part of the explanation for why stored malt displays better wort separation than newly kilned malt.

2.2. Ascorbate peroxidase

Kanauchi and Bamforth [19] examined ascorbate peroxidase in barley and germinated barley. The reaction of L-ascorbate peroxidase (EC 1.11.1.11; APX), an enzyme that exists in plants and microorganisms [20], is shown below.



It plays a role in removing damaging reactive oxygen species. This enzyme in barley grain has attracted little study [21].

No attention of researchers has been devoted to the relative importance of this enzyme in malting and brewing. Kanauchi and Bamforth [19] reported the effects on malting and brewing that are exerted by peroxidase functions.

Preparation of malt barley included its germination, as described by Hoy et al. [14]. The green malt was dried by lyophilization or by kilning at 60°C for 48 h. Barley or malt was ground in a food processor and was extracted by stirring on ice in 2.5 volumes of phosphate buffer (pH 7.0)-containing NaCl and EDTA. The slurry was then strained through cheesecloth. The resulting liquid was centrifuged at 4000× g to prepare a crude enzyme solution. Barley or malt was steeped in a hydrogen peroxide/ascorbic acid solution in phosphate buffer at 30°C for 1–4 h. After reaction, residual peroxide was detected using ferricyanide/ferric chloride or using a 3,35'-diaminobenzidine solution-containing peroxidase [22]. APX was assayed according to a procedure described by Nakano and Asada [23]. Ascorbic acid was assayed using an F-kit (Roche Diagnostics Corp.).

The development of APX in germinating barley started steeping immediately. It is extensive between the second and third days of germination. Thereafter, the levels start to decline. The enzyme level was raised considerably by the accompanying increased level of ascorbic acid (**Figure 1**). The enzyme is located primarily in the acrospire and the starchy endosperm (**Figure 2A–C**). Actually, the enzyme is abundant in the starchy endosperm. The enzyme survives light kilning (**Figure 3**). The activity of ascorbate peroxidase in germinated barley was 239.4 (U/g dry malt). After kilning, it was 159.6 (U/g dry malt) after 16 h; after 48 h, it was 136.5 (U/g dry malt). The enzyme reduces to 57.0–66.7% (data not shown).

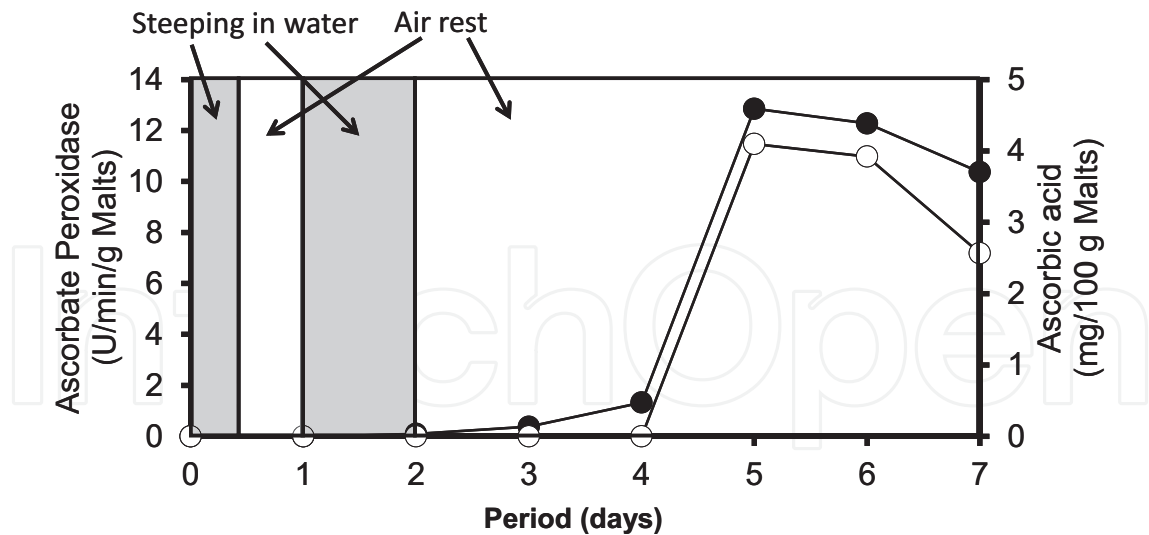


Figure 1. Levels of ascorbate peroxidase (●) and ascorbic acid (○) during the malting of barley.

To show the location of APX in kernels, endosperm was cut from sterile, dehusked barley at a distance of 2 mm. The slices were incubated at room temperature in sterilized gibberellic acid (10^{-5} M). After incubation, 5 mL of phosphate buffer (pH 7.0)-containing NaCl was added. Then the tissues were ground with a mortar and pestle. The extraction was prepared by centrifugation. A quantitative analysis of the distribution of the enzyme and also of ascorbic acid in the

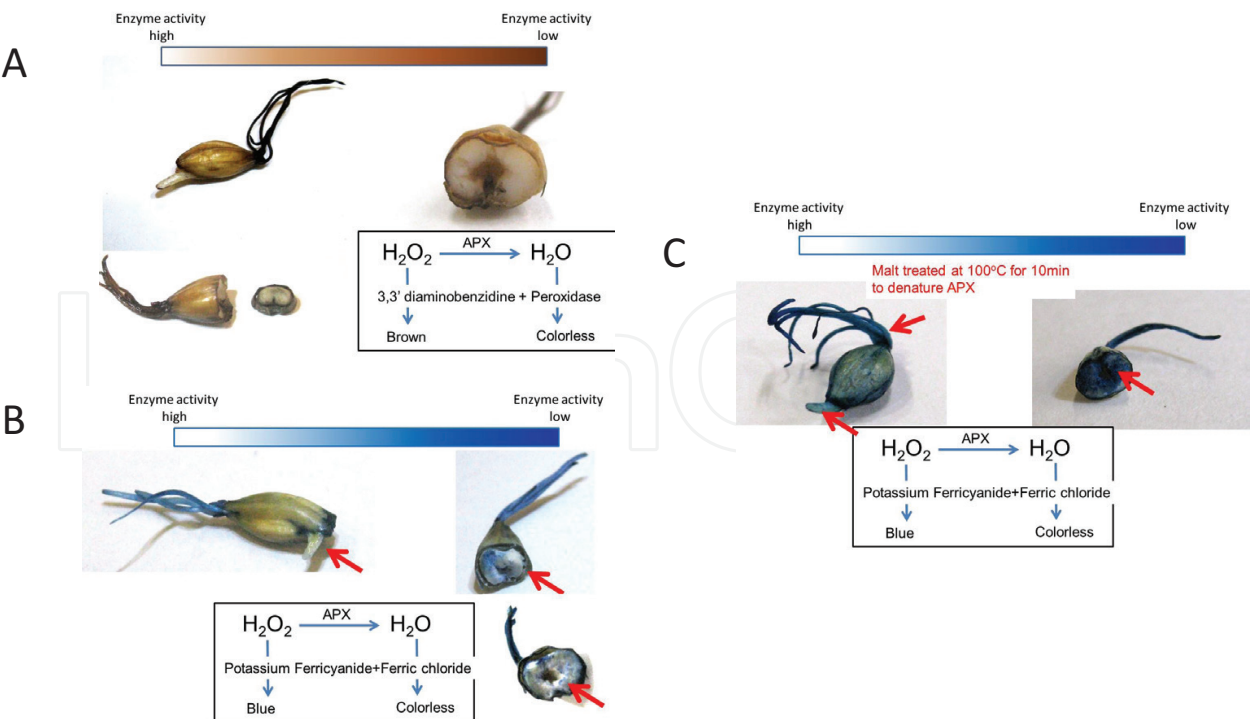


Figure 2. Detection of ascorbate peroxidase in sprouting barley (A) using 3,35'-diaminobenzidine as stain, (B) using potassium ferricyanide plus ferric chloride as stain, and (C) using potassium ferricyanide plus ferric chloride as stain on grain heated at 100°C for 10 min.

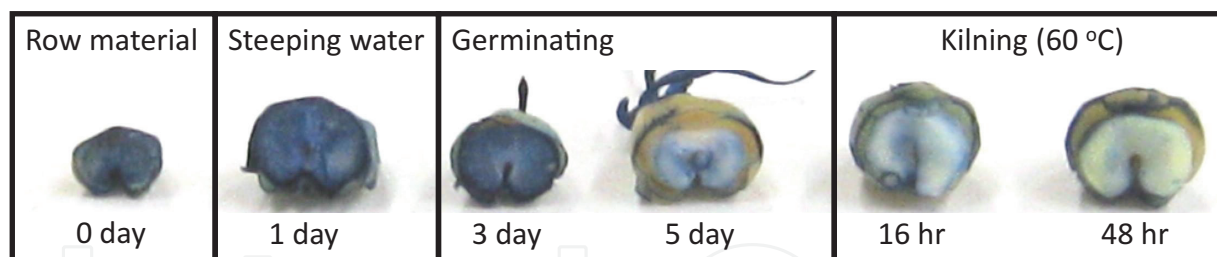


Figure 3. Detection of ascorbate peroxidase using potassium ferricyanide plus ferric chloride in half kernels of barley (A) sprouted for 1–6 days (B) in barley, during steeping, during germination, and after light kilning.

embryo, mid-grain, and distal regions of germinating barley is shown in **Figure 4A** and **B**. Each of the enzyme and ascorbic acid was detected in all three segments of the kernels after 2 days of germination. It is noteworthy that the mid-region has the highest levels of the enzyme and ascorbic acid. Kanauchi and Bamforth [19] inferred that these high levels reflect the development of enzyme in the acrospire growing under the husk, together with the development of the enzyme in the endosperm, presumably as a function of synthesis in the aleurone and rapid distribution into the cells of the starchy endosperm.

Synthesis of enzyme was attempted using inhibitors of RNA and protein and induction of a substrate. Inhibitors of RNA and protein synthesis, such as actinomycin D (80 $\mu\text{g}/\text{mL}$), 6-methylpurine (0.1 mM), puromycin (250 $\mu\text{g}/\text{mL}$), or cycloheximide (20 $\mu\text{g}/\text{mL}$), were added to barley during the development of ascorbate peroxidase. Actinomycin D and 6-methylpurine inhibited RNA synthesis. Furthermore, puromycin and cycloheximide

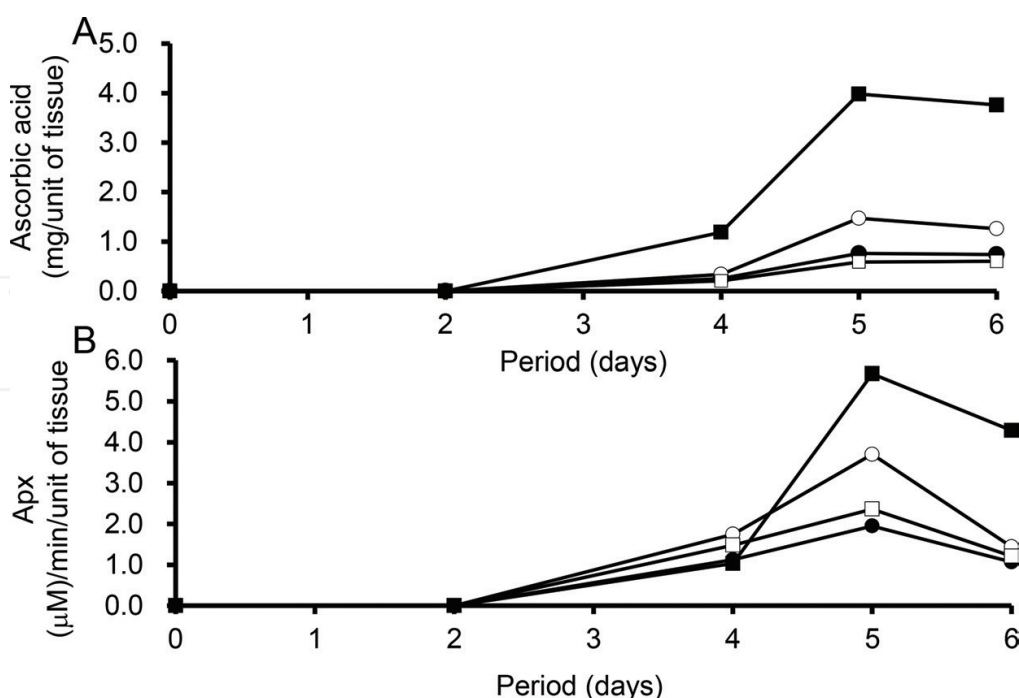


Figure 4. Incubation of barley slices (see **Figure 1**) in 10–5 M gibberellic acid solution: (A) levels of ascorbic acid; and (B) levels of ascorbate peroxidase. Embryo (●), endosperm 1 (○), endosperm 1 (□), and whole corn (■).

inhibited protein synthesis. Actinomycin D inhibited the development of new messenger RNA [24]. Puromycin did not inhibit enzyme development, but cycloheximide was a potent inhibitor. Kanauchi and Bamforth [19] concluded that the development of APX is a requirement for RNA and protein synthesis in the endosperm tissue.

The enzyme can also be induced by ascorbic acid and especially hydrogen peroxide and by a combination of the two. Results show that these are the active factors triggering the development of this enzyme (**Table 2**).

APX of crude extraction from malt was conducted by ammonium sulfate precipitate, chromatography using DEAE gel in column (25 mm × 300 mm), and size-exclusion column (10 mm × 350 mm, P-100 gel, Bio-Rad). It is apparently a monomer with relative molecular weight of approximately 26 kDa according to SDS-polyacrylamide gel electrophoresis. APX has an optimum pH of 5.5, with substantial activity at pH 5–7. Because the lower range pH encompasses the range of mash pH, the enzyme is reacted in mashing. However, it has very low capability to catalyze the removal of peroxide at pH 4–4.5, this condition meant that the enzyme reaction using an ascorbate for protection of beer against oxidation is extremely limited. Less than 50% of the activity survives heating at 50°C for 30 min. The enzyme is fundamentally destroyed in less than 10 min at 60°C (**Figure 5**).

Susceptibility of the enzyme to the chelating agents ethylenediaminetetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) suggests the necessity of a cation(s) for its full activity; but of the metal ions tested, only manganese exhibits any marked enhancement.

The enzyme is certainly inhibited by iron and copper, suggesting that the enzyme is inactivated by reactive oxygen species. Inhibition by azide suggests the presence of a functional heme group in the enzyme; indeed, heme has been shown to be the cofactor in this enzyme in other plants [25, 26]. Inhibition by iodoacetate indicates the functional presence of thiol groups, agreeing with others [25, 27]. 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 analyses were conducted according to Dalziel (**Table 3**) [28], revealing that the K_m values for ascorbic acid (ϕ_1/ϕ_0) and hydrogen peroxide (ϕ_2/ϕ_0) were 1.09 mM and 24.8 μM , respectively. Moreover, $V_{\max} (1/\phi_0)$ was 769 mM min^{-1} . The enzyme also had higher affinity for peroxide as opposed to ascorbate, which was true also for some other APX enzymes [26, 27].

	mU/10 slices	Error (mU)
Non	0.0	(+18.7)
Ultrafiltered extract	121.3	(+0.0)
5 mM H_2O_2	261.3	(+23.3)
5 mM ascorbic acid	46.7	(+39.7)
5 mM H_2O_2 + 5 mM ascorbic acid	280.0	(+14.0)

Table 2. Effects of hydrogen peroxide and ascorbic acid on the development of ascorbate peroxidase in barley slices.

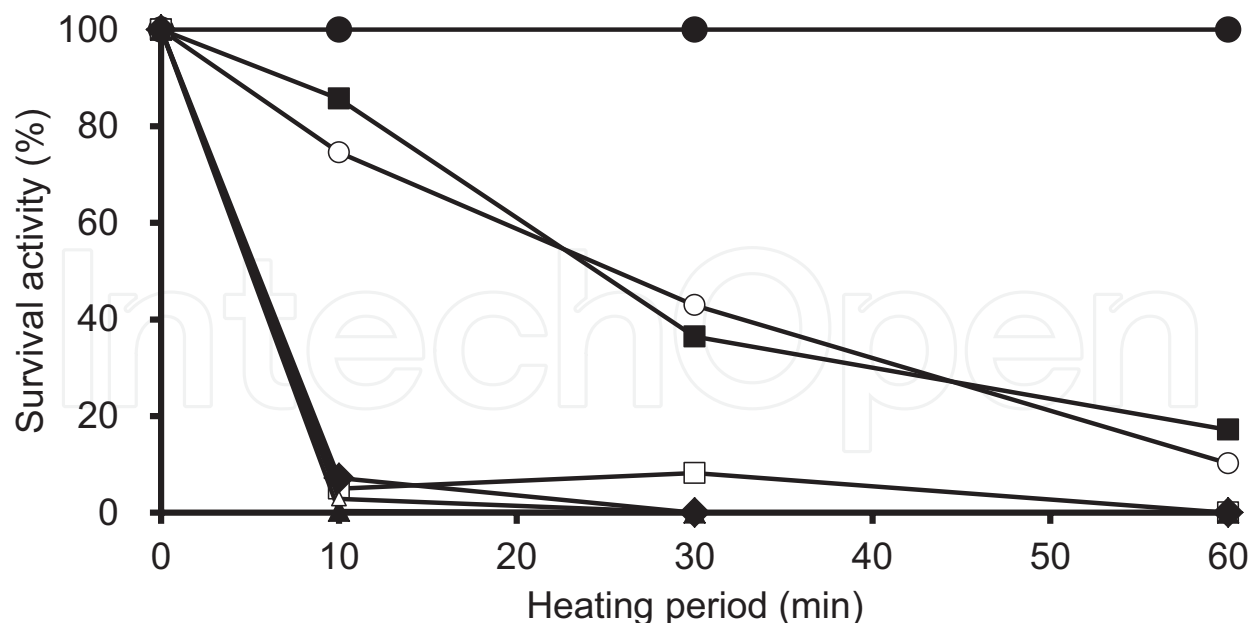


Figure 5. Heat tolerance of ascorbate peroxidase. Purified enzyme was heated at the temperatures indicated for 30 min before rapid cooling and subsequent assay. Symbols are 4°C (●), 40°C (○), 50°C (■), 60°C (□), 70°C (▲), 80°C (△), and 90°C (◆).

Ascorbate peroxidase was found in the acrospires and aleurones of germinating barley. Ascorbic acid and hydrogen peroxide induced the enzyme, which has a molecular weight of 23 kDa and a broad pH optimum, but which is sensitive to heat. Ascorbate peroxidase has very high affinity for hydrogen peroxide.

2.3. Ascorbic oxidase

Kanauchi et al. [29] reported the presence of ascorbic acid oxidase (AAO) and showed its properties.

Ascorbic acid oxidase (AAO EC 1.10.3.3) was found as “hexoxidase” in cabbage leaves in 1931 [30]. It catalyzes the following reaction.



φ0 (S)	φ1 (μM·S)	φ2 (μM·S)	φ12 (μM2·S)	φ1/φ0 (μM)	φ2/φ0 (μM)	φ12/φ2 (μM)	φ1φ2/φ12
0.0013	1.46	0.03	57.93	1089.8	24.76	1744.7	0.000837

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 (ascorbate). φ2/φ0 is the *K*_m for the secondary substrate (hydrogen peroxide).

Table 3. Kinetic parameters of ascorbate peroxidase.

AAO has been widely observed in various plant and fungal tissues [31–36]. Tamas et al. [37] reported AAO in germinating barley seed with the agent causing substantial inhibition of root-let growth. Honda [38] reported the enzyme's presence in barley roots and found it to be associated with cell walls. Kanauchi et al. [29] reported the existence in malt of a related enzyme: ascorbate peroxidase. Results show that this peroxidase has very high affinity for hydrogen peroxide and that the enzyme might have a valuable role in removing reactive oxygen species. However, the enzyme is heat-sensitive and therefore would not survive well during mashing.

Barley was germinated according to Hoy et al. [14]. AAO activity was assayed based on the measurement of the oxidation of ascorbate by the decrease in absorbance at 265 nm ($\epsilon = 14 \text{ mM cm}^{-1}$) at 25°C [39]. One unit of enzyme catalyzes the oxidation of 1 mM ascorbic acid (AA) per minute.

AAO is not present in ungerminated barley, but starts to be synthesized immediately upon steeping (**Figure 6**). It reaches a maximum level of activity early in germination, thereafter decreasing to a low but finite level at the end of germination. AAO was purified as follows. Crude extract was applied to a column of CM support gels. 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 precipitated using 80% saturation of ammonium sulfate. The precipitate was redissolved in citrate-phosphate (pH 7.0) and was then applied to the size-exclusion column. The eluent was citrate-phosphate.

After fractionation, crude extracts of malt by cation exchange chromatography revealed two peaks of AAO activity (data not shown). They are designated as AAO I and AAO II. Further chromatography of these peaks on Bio-Gel P100 (Bio-Rad) indicated that AAO I was of higher molecular size than AAO II. This characteristic was confirmed using polyacrylamide gel electrophoresis. Molecular weight estimates for the two enzymes are, respectively, 25–27 and 6–9 kDa. Two AAO enzymes were isolated from barley grains that are extremely different from any previously reported AAO [32–36]. Both enzymes are of much lower molecular size than previously

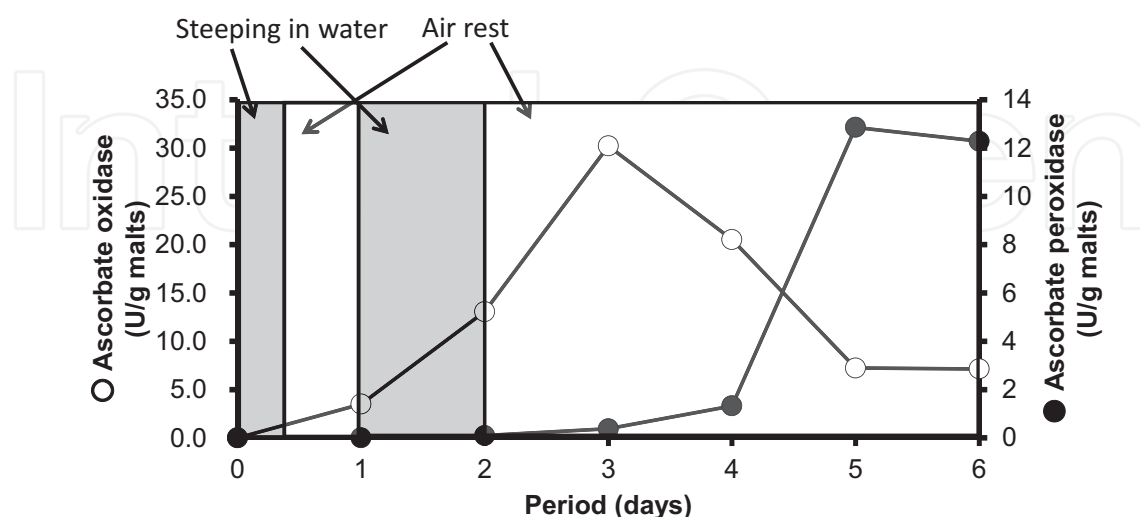


Figure 6. Levels of ascorbic acid oxidase and ascorbate peroxidase during steeping and germination of barley. Malt prepared at 15°C. Barley was sterilized using 1% calcium hypochlorite.

reported activities. AAO I had weakly cationic enzyme, with molecular weight of approximately 25 kDa. Furthermore, AAO II, which had a strongly cationic enzyme, had extremely low molecular weight of <10 kDa. The latter is one of the smallest enzymes ever reported: it is classifiable as a microenzyme [40, 41].

Optimum pH of AAO I and AAO II was pH 7.0. Furthermore, both activity pH had a broad pH range: AAO I has 66% relative activity; AAO II has 45% relative activity at pH 5.0. Furthermore, the optimum temperature of AAO I was 40°C, that of AAO II was 50°C. The enzyme displays slight activation by manganese and zinc. However, it is inhibited by copper, although AAO in most plants is generally described as an enzyme that is rich in copper. Iron, magnesium, and mercury inhibit them, especially AAO II. Furthermore, inhibition by chelating agents, such as EDTA and EGTA, is consistent with the need for a metal ion in the action of the enzymes. Inhibition by azide suggests the presence of a functional heme group in the enzyme. Inhibition by iodoacetate indicates the functional presence of thiol groups. Inhibition by N-bromosuccinimide suggests a role for tryptophan. Inhibition by benzenesulfonyl fluoride is consistent with a role for a serine group.

Kinetic parameters were calculated according to Dalziel [28] using the system shown in **Figure 7**. Substrate solutions (1 mL, 40°C) containing 0.0625–1.0 mM AA were introduced into

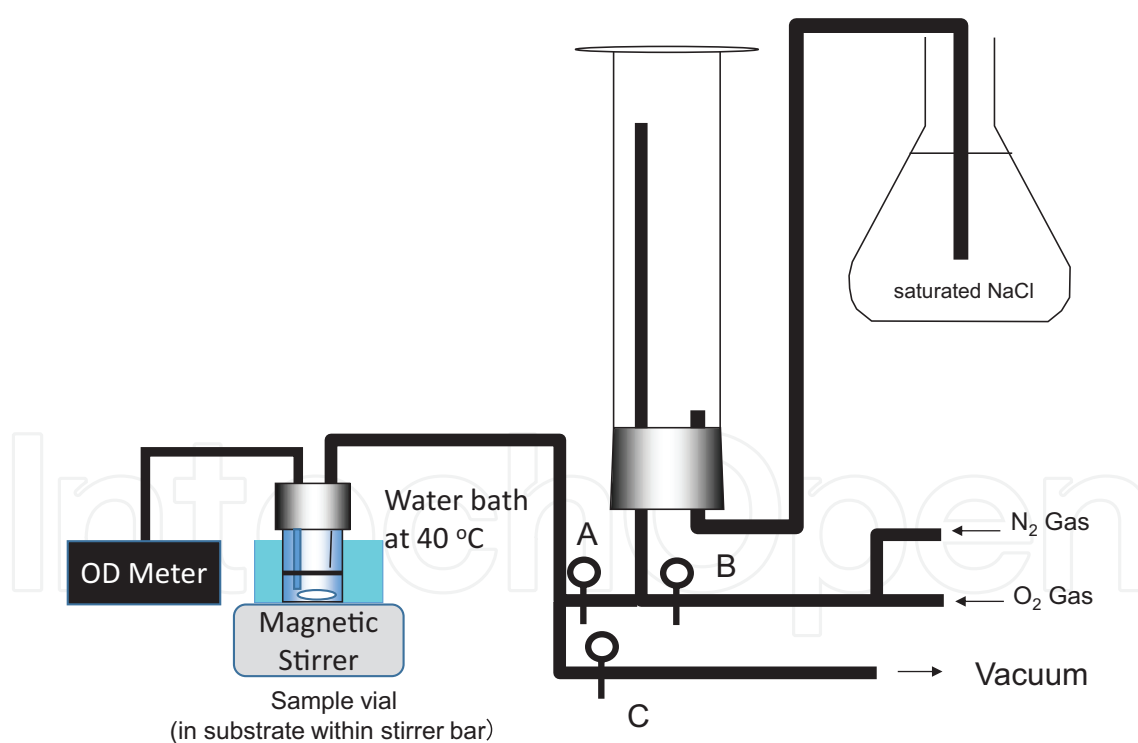


Figure 7. Apparatus for determining kinetic parameters for ascorbic acid oxidase. Air is removed from the vial using vacuum with stopcock C open. To adjust the oxygen concentration, a mixture of oxygen and nitrogen (e.g. 10 mL of O₂ and 90 mL of N₂) is transferred to the measuring cylinder filled with saturated NaCl solution via stopcock B. Upon closing B and opening stopcock A, the gas mixture is drawn vigorously to the vial from the 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.

a vial containing an electrode for measuring dissolved oxygen, which was then sealed with a rubber seal. A vacuum was applied in the vial. Then nitrogen gas, oxygen gas, and nitrogen-oxygen gas mixture (20–80% oxygen in nitrogen gas) were flushed successively through the vial. The operation was repeated three times. The enzyme solution was then added through the seal using a microsyringe. Decreased absorbance at 265 nm of the solutions was measured (Nano-Drop 2000; thermo Fisher Scientific Inc.).

Two substrate kinetic analyses (**Table 4**) revealed that AAO I can operate faster than $[V_{\max}]$ of AAO II. However, AAO II has much greater affinity for both substrates (lower Km values). The enzyme level declines as germination is prolonged. Actually, AAO II has much greater affinity (lower Km) for both substrates than AAO I does, although the latter displays a higher V_{\max} value. The Km value for AA displayed by AAO II is comparable with that reported for AAO from other organisms [32–38]. Few other papers report a Km value for oxygen, but the value we have measured for AAO II is comparable to that reported for *Acremonium* spp. [36].

Impact of AA addition during mashing was conducted as follows. Milled pale malt (50 g) was mashed at 65°C in a water bath with 150 mg of AA and 150 mL of deionized water. The mashes designated for 0 min were filtered immediately upon mixing. Subsequent mashes were sampled periodically at 10, 20, 40, and 60 min. Mashes were filtered through cone filters into an ice bath. Wort samples were cooled to 4°C and analyzed as soon as possible. Density, pH, color, total polyphenols, and free thiols in wort samples were determined. Mashes were performed at 65°C in the presence of 5.7 mM ascorbic acid (AA).

This value is well more than the km value for AAO II. Furthermore, the enzyme would be expected to operate at a maximum rate at the start of mashing (**Table 5**). Adding AA had little impact on the specific gravity of the recovered wort. Although the pH of the mash was lowered by AA initially, it rose progressively during mashing. The pH of the control mash decreased.

The addition of AA led to markedly higher levels of polyphenol and thiols being measurable in the wort.

This result is consistent with reports of AA functioning: AAO consumed oxygen, which was used to oxidize polyphenols and thiols. There is generally also a lower color observed in

	$\Phi 0$ (s)	$\Phi 1$ (mM s)	$\Phi 2$ (mM s)	$\Phi 12$ (mM ² s)	$1/\Phi 0$ (s ⁻¹)	$\Phi 1/\Phi 0$ (mM)	$\Phi 2/\Phi 0$ (mM)
AAO I	0.0012	0.0039	0.0129	0.0102	833	3.25	10.8
AAO II	0.0144	0.0051	0.0056	0.0105	69	0.35	0.39

The Φ parameters are calculated from secondary plots that are developed as described in the Materials and methods section. $\Phi 0$ 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. $\Phi 2$ is the slope of this line. $\Phi 1$ is the ordinate intercept of the plot of primary plot slopes against the reciprocal of the second substrate concentration. $\Phi 12$ is the slope of this line. $1/\Phi 0$ represents the true maximum velocity (V_{\max}). $\Phi 1/\Phi 0$ equals the Km for the primary substrate. $\Phi 2/\Phi 0$ is the Km for the secondary substrate. Ascorbic acid is the primary substrate and oxygen is the secondary substrate.

Table 4. Kinetic parameters of ascorbic acid oxidase (AAO).

Time (min)	pH	Specific gravity	Polyphenol (mg/L)	Thiols (A430)	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	5.385 ± 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

Table 5. Effect of ascorbic acid addition during mashing.

small-scale laboratory mashes (with the exception of the 60 min measurement, which was perhaps a spuriously high value). This result is regarded as consistent with less polyphenol oxidation in the mashes in view of the extremely high affinity of AAO II for AA and oxygen, coupled with its thermotolerance.

There are some consequences to oxygen ingress in a mash, including possibilities of oxidation of unsaturated fatty acids, cross-linking of thiol-rich proteins, and oxidation of polyphenols with the production of color [12]. The addition of AA to mashes is expected to engender diminution in such effects. Bamforth et al. [4] anticipated increased measurable levels of such groups in mashes containing AA. An increased level of polyphenol surviving into wort and a decrease in the amount of color produced would be expected.

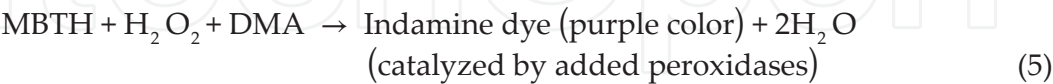
2.4. Oxalate oxidase

Oxalic acid can engender a range of problems in beer, including the blockage of dispensing pipes by beer stones, as well as turbidity and gushing [42, 43]. Importance of sufficient calcium to precipitate the material as calcium oxalate is notable. Few investigations have examined the origin of oxalic acid or of the enzymes that might produce or eliminate it during malting and brewing.

Oxalate oxidase (EC1.2.3.4) catalyses the conversion of oxalate into carbon dioxide and hydrogen peroxide. The hydrogen peroxide produced might serve some role in peroxidative cross-linking as part of cell wall restructuring [44]. The enzyme might also serve some role in countering the oxalate secreted by plant pathogens [45]. Oxalate oxidase is also known as germin [45, 46].

Kanauchi et al. [47] investigated oxalate oxidase of barley seed during malting and assessed its possible relevance in mashing. Barley was germinated according to Hoy [14]. The barley was germinated at 16°C for 6 days. The green malt was dried at 60°C for 16 h to approximately 5% moisture. The rootlets were not removed.

Oxalate oxidase was assayed according to the following principle [48].



where MBTH is 3-methyl-2-benzothiazolinone hydrazone; DMA is N,N-dimethylaniline.

Enzyme solution was mixed with oxalic acid solution (pH 4.0), MBTH solution, N,N-dimethylaniline, 3-methyl-2-benzothiazolinone hydrazone, ethylenediaminetetraacetic acid (EDTA) solution, and peroxidase enzyme solution. The standard curve was produced using hydrogen peroxide. One unit (1 U) of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of substrate per minute under assaying conditions.

Its location in the grain remains as a matter for speculation. Oxalate oxidase was present in unmalted grain. Its activity increased slightly during germination (**Figure 8**). In germinated grain, 61% of the oxalic acid was in the rootlets, 32% in the acrospires, and the balance in the bran (husk/pericarp-testa/aleurone). Oxalic acid was not found in the starchy endosperm. It is noteworthy that more than half of the oxalate is located in the rootlets, which are removed

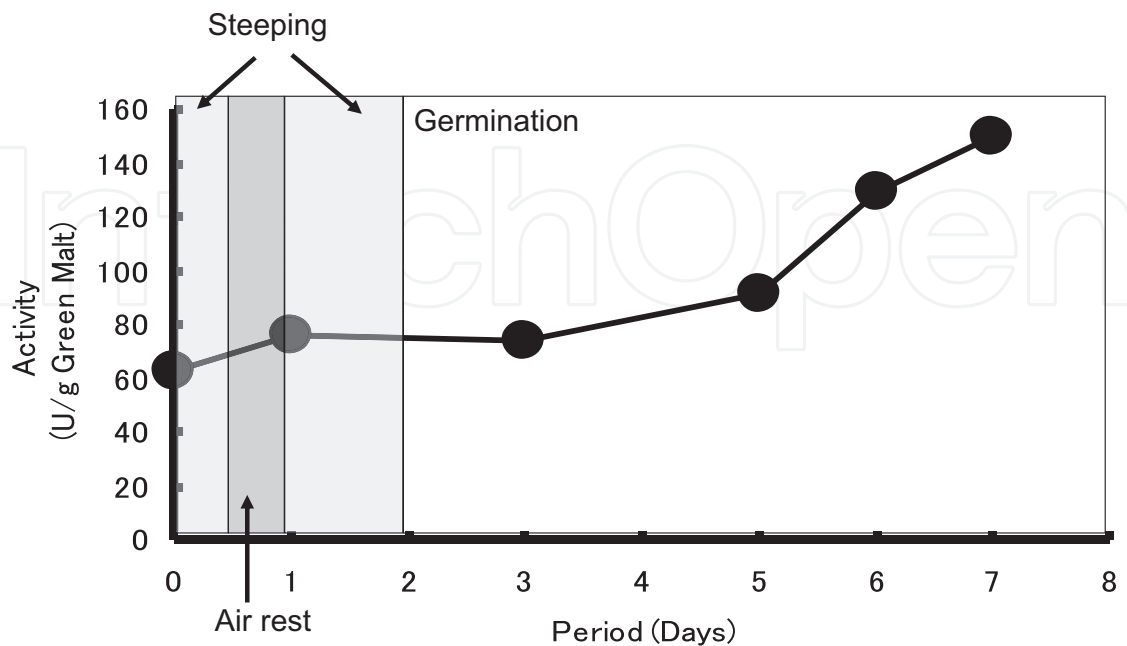


Figure 8. Oxalate oxidase activity of Starling barley during malting.

during malt production. Oxalic acid was not detectable in barley. Concentrations of oxalate in kernels increased during germination, although the amounts decreased near the end of germination. According to activity dyeing, the enzyme is present in the aleurone and the embryo, but not in the endosperm (**Figure 9**).

Ground-germinated grain was extracted in two volumes of citrate-phosphate buffer. After centrifugation of slurry, the extract was purified by precipitation using ammonium sulfate, a DEAE column, and a size-exclusion column. Consequently, the enzyme was partially purified using ammonium sulfate precipitation, ion exchange chromatography, and gel permeation chromatography, with a finishing specific activity of 44.6 U/mg protein (19.6-fold purified). The molecular weight of the enzyme was 58.5 kDa as determined from SDS-PAGE. Others reported an oxalate oxidase of rootlets of barley, which is a pentamer with subunit molecular weight of 25 kDa [49].

The enzyme has a pH optimum of approximately 4.0, but it displayed 90% of the maximum activity at pH 3 and 40% of the maximum activity at pH 8.0. Two substrate kinetic analyses, which showed K_m values for the oxalate was 0.1 mM, K_m values for oxygen was 0.46 mM. V_{max} value for oxalic acid were 18.2 mM/min and its value for oxygen was 285.7 mM/min.

Several metal ions influenced the activity of oxalate oxidase (data not shown), such as manganese which slightly activated the enzyme. Generally, a manganese-claiming enzyme is a manganese-containing enzyme [50]. However, zinc and copper activated the enzyme to a greater degree. The enzyme was inhibited by cobalt, by iron, and to a lesser extent by magnesium. Inhibition by mercury, dithiothreitol, and iodoacetamide suggests that the enzyme needed free thiol groups for activity. This result was confounded by the observation that N-ethylmaleimide did not inhibit its activity. The enzyme was inhibited by azide. It was activated by flavin adenine dinucleotide (FAD), and reportedly might be a flavoprotein.

Kanauchi et al. [47] reported that barley kernels contain oxalate oxidase located in the living tissues of roots, but not in the starchy endosperm. Because the enzyme is active in a broad pH range, and because it has high heat tolerance, it was active during mashing, but it was less important than other oxidases for scavenging oxygen from mashes because of its low affinity for oxygen.



Figure 9. Location of oxalate oxidase in grain. Blue coloration denotes enzymic activity. The top photographs show germinated grain, whereas the bottom photograph shows unmalting grain.

3. Conclusion

Malt contains some enzymes or characteristics that degrade beer quality. This chapter presents descriptions of them. Four oxidations were investigated. The enzymes in malt have heat stability. Its optimum pH (pH 8.0) shows that the enzyme limits the reaction in the mash at pH 5.0. The activity of thiol oxidase lessens during malt storage. Results suggest that this is part of the explanation for why stored malt displays better wort separation than newly kilned malt.

Ascorbate peroxidase was identified in the acrospires and aleurones of germinating barley. Its synthesis was induced by the presence of ascorbic acid and hydrogen peroxide. It was found to have a molecular weight of 26 kDa and a broad pH optimum: pH 5–7. However, the enzyme lost 50% of its activity in 30 min at 40°C. The enzyme has very high affinity for hydrogen peroxide.

Ascorbic acid oxidase(AAO), which is an antioxidant agent as ascorbic acid, is developed in embryo tissues of barley during steeping and during the initial stages of germination. The molecular weights of the two AAO enzymes in malt are 27.4 and 6.4 kDa, respectively; their optimum pH is 7.0 and their optimum temperature is 40–50°C. Both enzymes are extremely heat tolerant and are capable of acting over a broad pH range. These two enzymes are expected to function during conversion temperatures of mashing.

Addition of ascorbic acid to mashes results in the survival of higher levels of polyphenol and thiols into wort and reduced color in that wort, commensurate with AAO preferentially consuming oxygen. Consequently, in adding ascorbic acid, oxygen is less available for other reactions, such as thiol oxidation and polyphenol oxidation in mashes.

Oxalate oxidase in barley kernels is located in living tissues of roots. The enzyme had active in a broad pH range, and it has high thermal tolerance. However, it is probably less important than other oxidases in scavenging oxygen from mash, because of its affinity for oxygen was low.

As explained herein, beer quality can be improved by the regulation of dissolved oxygen and oxidation enzymes, such as thiol oxidase, AAO of the endosperm cell walls in malt.

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