DETERMINATION OF FATTY ACID HYDROPEROXIDES PRODUCED DURING THE PRODUCTION OF WORT

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Linoic acid and linolenic acid hydroperoxides in malt, mash, or wort were determined with high sensitivity and high selectivity by the chemiluminescence-high performance liquid chromatography (CL-HPLC) method using isoluminol-microperoxidase solution as a luminescing reagent. The determination limit of this method for both hydroperoxides was 0.1 μM in mash or wort. During the mashing in a laboratory mash bath, the hydroperoxides started to increase just after mashing-in, reached a maximum at 65°C, and then decreased. Though the hydroperoxides were detected in mash just before the lautering in a pilot scale brewing, they disappeared during the lautering and could not be detected during the subsequent stages of wort production. Therefore, it was thought that the mashing process is the most important of the lipid oxidation reactions during wort production. It is also expected that the CL-HPLC method can give useful information on lipid oxidation mechanisms during wort production.

Key Words: Linoleic and linolenic acid hydroperoxides, Chemiluminescence-HPLC, determination, malt, mash, wort

INTRODUCTION
Fatty acids in wort and beer are important because they affect several beer qualities and yeast metabolism. Wort lipid including fatty acids is necessary for activation of yeast cell growth and significantly affects the fermentation process. The ratio of unsaturated (inhibitor) and saturated (promotor) acids is related to gushing problems. It has been shown that long-chain fatty acids (C_{12}-C_{18}) have a relationship with beer head retention. Some fatty acids have a high flavour potential. Especially, linoleic and linolenic acids have received great attention because their oxidative degradation may lead to the formation of a characteristic aging flavour.

The enzymatic- or auto-oxidation of these fatty acids results in the formation of their hydroperoxides as primary products. The hydroperoxides are unstable and degrade into low molecular weight compounds (aldehydes, ketones, and acids) that include 2-alkanals and 2,4-alkadienals. Some of these carbonyls are responsible for aged flavour in beer. The enzymatic oxidation is caused by lipoygenase in malt during the wort making process and the auto-oxidation occurs during both wort making and beer storage. The oxidation products involving several carbonyls survive in the finished beer, thus contributing to the aging off-flavour during beer storage. However, it has not been fully elucidated whether enzymatic- or auto-oxidation contributes more to the oxidation of fatty acids during wort making. One of the reasons is that a suitable determination method of the hydroperoxides in mash or wort has not been developed and that their behaviour during the wort making process is still unknown.

The measurement of lipid oxidation is associated with a number of serious practical problems. The most commonly used methods such as the thiobarbituric acid (TBA) assay and the determination of conjugated dienes lack specificity. The TBA assay measures only a small fraction of the aldehydic breakdown products, mainly malonaldehyde, of those fatty acid hydroperoxides that contain three or more double bonds. Measurement of conjugated dienes is not specific for fatty acid hydroperoxides but detects the corresponding fatty acid hydroperoxides with nearly the same sensitivity.

This method is also interfered with by several components in mash, wort and beer which have UV absorbance at 234 nm. Recently, a chemiluminescence-high performance liquid chromatography (CL-HPLC) system was developed by Miyazawa et al. and Yamamoto et al. for the sensitive and selective detection of the hydroperoxides of lipids. This method is based on detecting chemiluminescence generated during the oxidation of luminol or isoluminol by the reaction of hydroperoxides with cytochrome c-heme.

In this paper, a suitable determination method for fatty acid hydroperoxides in mash or wort was established using the CL-HPLC method, and the behaviour of linoleic and linolenic acid hydroperoxides during wort production were studied.

MATERIALS AND METHODS

Chemicals
Isoluminol (6-amino-2,3-dihydro-1,4-phtalazinedione) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Microperoxidase (MP-11), soybean-lipoxygenase (type I), linoleic acid, and linolenic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 9(S)-hydroperoxy-(10E,12Z)-octadecenoic acid and 13(S)-hydroperoxy-(9Z,11E)-octadecenoic acid were purchased from Cascade Biochem Ltd (Tokyo, Japan).

Preparation of linolenic acid hydroperoxide
Linolenic acid hydroperoxide was prepared by the reaction of linolenic acid standard with soybean lipoxygenase. The hydroperoxide was purified by thin-layer chromatography.

Apparatus
The HPLC instrument was a Waters 600E system controller (Waters, Millford, MA) equipped with a Waters 700 WISP automatic sample injector, a Waters 486 tunable absorbance detector, a column thermostatting oven, a chemiluminescence detector (Soma Kogaku, Tokyo, Japan) and a Waters M741 data module. The analytical column was a μ-Bondapak C_{18} (3.9 × 300 mm, Waters).

Wort production on a pilot scale
In a 400 litre scale pilot plant, wort making was carried out by the two mash method using about 30% of adjuncts (Starch, rice and corn grits). Mashing started at 50°C and saccharification was carried out at 65°C. Natural hop pellet was used at the wort boiling process.

Mashing in a laboratory mash bath
Malt was milled using a Buhler-miag mill (0.2 mm, Minneapolis, MN) and 70.6 g of the meal was incubated with 200 ml of water in a laboratory mash bath. A temperature
programme for the mashing in the mash bath is shown in Figure 7.

Sample preparation
Mash or wort (100 ml) were cooled by the addition of 50 ml cold distilled water (0°C) in an ice bath. Cold mash (150 ml) and 150 ml of ether-pentane (E-P) solution (1:1) were transferred into a 500 ml separation funnel and were shaken for 10 min. The E-P phase (8 ml) was evaporated under a N₂ atmosphere. The residue was dissolved in 2 ml of methanol, and the solution was filtered using a 0.45 µM membrane filter.

Preparation of luminescing reagent
Microperoxidase (6.25 mg/litre) and isoluminol (44.3 mg/litre) were dissolved in 100 mM borate buffer-70% methanol solution (pH 10.0). This solution was prepared daily.

CL-HPLC conditions
CL-HPLC conditions were as follows: eluting solution, 80% methanol containing 0.07% acetic acid; flow rate, 1.0 ml/min (eluting solution), 1.8 ml/min (luminescing reagent); column temperature, 25°C; chart speed, 2 mm/min; injection volume, 10 µl. Both the eluting solution and luminescing reagent were purged with helium gas during analysis to exclude dissolved oxygen in the solutions.

RESULTS AND DISCUSSION

Determination of hydroperoxides in mash and wort
The principle of the method is as follows; hydroperoxides separated by the HPLC column are mixed with luminescing reagent and the chemiluminescence produced in the reaction is detected. Hydroperoxides react with heme-peptide (cytochrome C or microperoxidase) and produce peroxyradical or oxidants which produce active oxygen via the Russel reaction (2ROO• → O₂ + ROH + RC = O). The active oxygens oxidize luminol or isoluminol under alkaline conditions and generate chemiluminescence. The CL-HPLC method has high sensitivity and selectivity, and is the most reliable analysis for hydroperoxides. It has been used for the determination of lipid hydroperoxides in biological samples such as human blood plasma, lipoprotein fractions, tissue organs of experimental animals and cultured human fetal diploid cells 7.

Figure 1 shows the chromatograms of the linoleic and linolenic acid hydroperoxides. Fatty acid hydroperoxides were separated using a reverse phase HPLC column. Acetic acid was added to 80% methanol (final concentration 0.07%) as an ion-paired reagent, which gave better separation, sharper peaks for the hydroperoxides, and more stable retention times. The pH of the luminescing reagent was not affected by this addition.

When cytochrome C-luminol solution, microperoxidase-luminol solution, or microperoxidase-isoluminol solution was used as a luminescing reagent, the sensitivity of the cytochrome C-luminol solution was not satisfied, and the microperoxidase-luminol solution showed a high background and noise level. The microperoxidase-isoluminol solution gave sharper peaks and higher sensitivity of hydroperoxides and a more stable background on the chromatograms. 9(S)-hydroperoxy-(10E,12Z)-octadecenoic acid (9-LOOH) and 13(S)-hydroperoxy-(9Z,11E)-octadecenoic acid (13-LOOH), which are isomers of linoleic acid hydroperoxides, showed the same retention time in this chromatogram.

A good linear relationship between the concentration of standard hydroperoxides and their peak area was obtained by the present CL-HPLC method (Fig. 2). The detection and determination limits of linoleic and linolenic acid hydroperoxides were 10 picomol and 1 picomol, respectively. The CL detection sensitivity was much higher than the UV (234 nm) detection.
Hydroperoxides, the chemiluminescence peaks 1 and 2 completely disappeared, while the UV absorbance (234 nm) peaks a and b increased slightly (Fig. 5). It is likely that the chemiluminescence peaks showed some hydroperoxides, but the UV peaks represent not only hydroperoxides but also other materials. Chemiluminescence peaks 1 and 2 seem to show linoleic acid hydroperoxide and linolenic acid hydroperoxide, respectively.

The chloroform-methanol (C-M) (1:1, v/v) extraction method has been generally used for the extraction of lipid hydroperoxides in food and biochemical samples. Some materials in the C-M extract from mash quenched the chemiluminescence and appeared as negative peaks, which interfered with the determination of hydroperoxides. When the hydroperoxides were extracted quantitatively with ether-pentane (E-P) (1:1, v/v) containing 0.002% butylated hydroxytoluene as an antioxidant, the interference disappeared on the chromatogram, and sharp peaks of linoleic and linolenic acid hydroperoxides were obtained (Figs. 3-5). The efficiency using the E-P extraction was more than 85%.

Figure 6 shows the calibration curves of linoleic and linolenic acid hydroperoxides in mash and wort. The calibration graphs prepared by the standard addition method were linear over 0-20 μM added hydroperoxides. Their determination limits were both 0.1 μM. Based on the results so far, it was shown that the CL-HPLC method after the E-P extraction is the most useful analysis of linoleic and linolenic acid hydroperoxides in malt, mash, or wort.

**Behaviour of hydroperoxides produced during wort making process**

Schooner Malt contained 0.4 μM hydroperoxides of linoleic acid and a trace level of linolenic acid (Fig. 3). During wort production in a pilot plant, the concentration of linoleic acid hydroperoxide was 11.2 μM after 10 min at 50°C and decreased to 4.3 μM before lautering, but was not detected from lautering to boiling (Table I). The behaviour of linolenic acid hydroperoxide showed the same tendency. The level of linoleic acid hydroperoxide was about six times that of linolenic acid hydroperoxide. It appears that hydroperoxides formed by the oxidation of unsaturated fatty acids are mainly produced during mashing and are hardly produced in wort after lautering, because lipids including unsaturated fatty acids and hydroperoxides are insoluble in wort and most of them are excluded by lautering. Figure 7 shows the behaviour of linoleic and linolenic acid hydroperoxides during mashing in a laboratory mash bath. Both hydroperoxides started to increase just after mashing-in, reached a maximum after 10 min at 65°C and then decreased. In the hydroperoxides curves shoulders were observed around 10 min at 48°C. The production level of linoleic acid hydroperoxide was about six times more than that of the linolenic acid hydroperoxides. The levels of hydroperoxides produced in the laboratory mash bath were higher than those in the pilot plant mash. It is thought that the difference was caused by the malt ratio.

**TABLE I. Changes in contents of hydroperoxides produced during wort making process on 400 litre pilot scale**

<table>
<thead>
<tr>
<th></th>
<th>hydroperoxides (μM) of linoleic acid</th>
<th>linolenic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>mash (at 50°C, 10 min)</td>
<td>11.2</td>
<td>1.7</td>
</tr>
<tr>
<td>mash (before lautering)</td>
<td>4.3</td>
<td>0.8</td>
</tr>
<tr>
<td>wort (after lautering)</td>
<td>n.d.*</td>
<td>n.d.</td>
</tr>
<tr>
<td>wort (after boiling)</td>
<td>n.d.</td>
<td>n.d.</td>
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*no detect
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in mashing; all malt was used in the laboratory mashing, while starch, rice, and corn grists were used with malt in the pilot plant mashing.

It has been reported that the linoleic acid hydroperoxides are transformed to some extent into the corresponding α- and γ-ketols by an isomerase during mashing and these hydroperoxides and ketols are transformed into mono-, di-, trihydroxy-, and hydroepoxy acids by chemical reactions. The hydrophilic dihydroxy- and trihydroxy acids are then transferred to beer. The vicinal di- and vicinal trihydroxy-acids are split into flavour active carbonyls such as hexanal and nonenal. In the analogous reaction, the corresponding dihydroxy acids from linolenic acid are transformed into hexenal and nonadienal. It has been shown that the level of the trihydroxy octadecenoic acid produced during mashing is about 15 ppm, which was of a similar order of magnitude with linoleic acid hydroperoxide in this study.

In conclusion, the behaviour of hydroperoxides during wort production could be clarified by the CL-HPLC method. The oxidation mechanisms of lipids during wort production will be explored by detailed studies using this new analysis.

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REFERENCES