Determination of Endogenous Gibberellins in Germinating Barley by Combined Gas Chromatography-Mass Spectrometry[1]

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ABSTRACT

This study was undertaken to elucidate the dominant endogenous gibberellins (GA) and the changes in their levels in germinating barley during malting. Also, a method for determining gibberellins in barley and malt by combined gas chromatography-mass spectrometry (GC-MS) was developed. Endogenous gibberellins in germinating Fuji Nijo II and Betzes barley were sequentially extracted with ethanol and ethyl acetate. Fractions corresponding to GA_1, GA_3, GA_4, and GA_7 were separated and collected from the ethyl acetate extract by Sephadex LH-20 column chromatography, silicic acid partition column chromatography, silica gel thin-layer chromatography, and kieselguhr thin-layer chromatography. Biological activity of each fraction was examined by bioassay with dwarf rice seedlings (var. Tan-ginbozu) and deembryonated barley endosperm. Identification of each gibberellin was performed by analysis of the methyl ester trimethylsilyl ether derivative of gibberellin in each fraction by GC-MS. Determination of gibberellin content was performed by mass fragmentography with GC-MS. The data indicated that the dominant gibberellin in germinating barley is GA_1 and that GA_3 is a minor gibberellin. GA_4 and GA_7 were not identified. GA_1 and GA_3 contents in germinating barley under malting conditions reached their maximum concentrations on the second day; levels decreased on the fourth and sixth days of germination. The behavior of authentic GA_3 added to steeped barley during germination and the contents of residual GA_3 in commercial malts were also examined.

Key words: *Barley, Gas chromatography-mass spectrometry, Germination, Gibberellin, Mass fragmentography*

When barley germination begins during malting, gibberellins (GA) are secreted from the embryo (24,33,37), inducing or activating varied hydrolytic enzymes, such as α-amylase, endo-β-glucanase, and peptidase, which accelerate the modification of endosperm (20,29). Certain factors, such as pressure treatment during the steeping process, restrict the secretion of endogenous gibberellins from the embryo, resulting in poor malt quality (38). When exogenous GA_3 is added to the barley after the embryo has been damaged by physical or chemical methods, a malt of good quality is obtained (17,30). Exogenous GA_3 is used to increase extract yield and shorten malting time. For these reasons, the presence of gibberellins is an extremely significant factor in the malting process. In studies of the physiology of barley germination to improve malt quality, understanding the behavior of endogenous gibberellins in barley kernels is very important; however, the determination of endogenous gibberellins in barley kernels at the germination stage is difficult because of their extremely low levels. Bioassay with certain plant tissues sensitive to small amounts of gibberellins, such as barley endosperm (1,5,33,38) lettuce hypocotyl (6), and Rumex leaf (33), is most often employed in studies of endogenous gibberellins. Isotopic dilution procedures (18,19,22), an effective method for determination of very small quantities of certain compounds, is also used. On the basis of studies using these methods, two theories have emerged on the sort of dominant endogenous gibberellin in germinating barley kernels. GA_1 (33) and GA_3 (5,6,18,19,22) are suggested as the dominant gibberellins by these theories. The GA_3 concept has been most widely accepted.

In this study, identification of endogenous gibberellins was performed by combined gas chromatography-mass spectrometry (GC-MS). Also, a new, highly sensitive method was developed for the determination of gibberellins in barley and malt by mass fragmentography with GC-MS. Changes in the levels of endogenous gibberellins in germinating barley during malting were examined by this method. The behavior of authentic GA_3 added to steeped barley during germination and residual GA_3 content in commercial malt were also determined by this new method.

[1]Presented at the 47th Annual Meeting. Miami, FL, May 1981.

0361-0470/82/01001808/$3.00/0

EXPERIMENTAL

Barley Samples

Betzes (two-row) barley imported from Canada (1978) and Fuji Nijo II (Japanese two-row barley), cultivated in Yamanashi Prefecture (1978), were used. Samples were graded, and kernels remaining on a 2.5-mm sieve were used. All barley samples had a good appearance and none were dormant.

Authentic Gibberellins

GA_1 was provided by Prof. Takahashi of the University of Tokyo. GA_3 was purchased from Tokyo Kasei Co., Ltd., and a mixture of GA_4 and GA_7 was obtained from Kyowa Hakko Co., Ltd.

Malting and Sampling Method

Malting was done in our small-scale plant. Barley samples were steeped in running water (4 L/min/30 kg of barley) to 43% moisture content. The steeped barley was transferred to a germination box, and the temperature of the germinating barley was raised from 14 to 18°C at the rate of 1°C per day. When a temperature of 18°C was attained, it was maintained at that temperature for two days. The total germination period was six days. The germinating barley was turned by hand daily. Sampling of the germinating barley for gibberellin analysis was performed at the end of steeping and on days 2, 4, and 6 during germination. Germinating barley samples were stored without drying at −25°C until analysis.

Extraction and Purification of Endogenous Gibberellins from Germinating Barley

Extraction. Two kilograms of germinating barley was divided into four parts. Each part was homogenized in 2 L of ethanol with a Polytoron (type PT 20 ST "OD" SM, Kinematica G.m.b.H., Switzerland). The homogenate was agitated for 3 hr and allowed to stand overnight at 8°C. The extracts were centrifuged at 4,300 × g for 15 min. The clear supernatant obtained was decanted and put aside. After ethanol in the extract was removed by rotary evaporation at a temperature below 30°C, 50 ml of water was added to the residue and the pH of the aqueous solution was adjusted to 9.0 by addition of 1N NaOH. Basic and neutral materials were extracted with ethyl acetate (once with 80 ml, three times with 50 ml), and the ethyl acetate fraction was discarded. The aqueous phase was adjusted to pH 2.5 with 10% phosphoric acid solution, and acidic materials containing gibberellins were then extracted with ethyl acetate (twice with 50 ml, three times with 25 ml). The ethyl acetate fractions were combined and freeze-dried. The solvent was removed in vacuo.

Sephadex LH-20 Column Chromatography. The residue was dissolved with a small portion of acetone (2–3 ml), charged on Sephadex LH-20 (Pharmacia Fine Chemicals AB, Sweden)

column (30 × 3 cm) and eluted with acetone. The eluate (200–440 ml) was collected and the solvent was removed in vacuo.

Silicic Acid Partition Column Chromatography. Thirty grams of silicic acid (Mallinckrodt, 100 mesh) was hydrated with 18 ml of 0.5*M* formic acid, slurried with *n*-hexane:ethyl acetate (90:10) saturated with 0.5*M* formic acid, and poured into a column with a diameter of 2.5 cm. The materials separated by Sephadex LH-20 column chromatography were dissolved in 1 ml of ethyl acetate. Next, 9 ml of *n*-hexane was added to the ethyl acetate solution. The resultant suspension was charged on a silicic acid column and eluted with 150 ml of *n*-hexane:ethyl acetate (90:10) saturated with 0.5*M* formic acid. The eluate was discarded. The elution of the column was continued with 200 ml of *n*-hexane:ethyl acetate (60:40) saturated with 0.5*M* formic acid. The eluate (60% *n*-hexane fraction) was put aside. The solvent was changed to *n*-hexane:ethyl acetate (20:80) saturated with 0.5*M* formic acid, and the column was eluted until 200 ml of eluate (20% *n*-hexane fraction) was obtained. The solvents of both fractions were removed in vacuo.

Preparative Thin-Layer Chromatography. The residues of the 60% *n*-hexane fraction and the 20% *n*-hexane fraction obtained by silicic acid partition column chromatography were dissolved in a small amount of ethyl acetate and streaked with a micropipet in a 12-cm band on a 20 × 20-cm silica gel 60 plate 2 mm thick (Merck, Germany). On both sides of the 60% *n*-hexane fraction band, appropriate amounts of authentic GA_4/GA_7 were similarly spotted. Authentic GA_1/GA_3 was used for guide spots with the 20% *n*-hexane fraction band. The plate on which the 60% *n*-hexane fraction was applied was developed with chloroform:acetic acid (95:5), and that containing the 20% *n*-hexane fraction was developed with ethyl acetate:chloroform:acetic acid (20:8:1). After development, each plate was air dried and 70% sulfuric acid was sprayed on the guide spots to determine the location of GA_4/GA_7 and GA_1/GA_3 by ultraviolet light examination. The zones corresponding to GA_4/GA_7 and GA_1/GA_3 were scraped from the plates and eluted with 150 ml of methanol by agitating 1 hr. The methanol extracts were concentrated to dryness in vacuo.

Separation of Fractions Corresponding to GA_1 and GA_3 by Thin-Layer Chromatography. The residual matter corresponding to GA_1/GA_3 separated by preparative thin-layer chromatography was applied to a kieselguhr F254 plate 20 × 20-cm and 0.2 mm thick (Merck, Germany) as for preparative chromatography. The plate was equilibrated for 3 hr in lower phase vapor of benzene:ethyl acetate:acetic acid:water (55:25:30:50) and developed with upper phase. The guide spots were removed with a glass cutter. Cut plates were heated to 100° C for 10 min after applying 70% sulfuric acid by spray, and the locations of GA_1 and GA_3 were detected by ultraviolet examination. The zones corresponding to GA_1 and GA_3 were scraped from the plate and the scraped kieselguhr was then agitated with 25 ml of water for 1 hr and adjusted to pH 2.5 by the addition of 10% phosphoric acid solution. Acidic materials were extracted with ethyl acetate (25 ml, four times) and the solvent was removed in vacuo after dehydration with anhydrous sodium sulfate.

Separation of Fractions Corresponding to GA_4 and GA_7 by Thin-Layer Chromatography. The residual matter corresponding to GA_4/GA_7, separated by preparative thin-layer chromatography, was applied to a 20 × 20-cm and 0.25-mm thick silica gel 60 plate with the concentration zone (Merck, Germany). The plate was developed with ethyl acetate, and the zones corresponding to GA_4 and GA_7 were detected in the same manner as for preparative chromatography. These zones were scraped from the plate and extracted with methanol, and the solvent was removed in vacuo.

Extraction and Purification of Residual GA_3 from Commercial Malt

One hundred grams of finely ground malt was homogenized in 560 ml of 90% ethanol with a Polytoron. The homogenate was agitated for 3 hr and allowed to stand overnight at 8° C. The extracts were centrifuged at 4,300 × *g* for 15 min. The clear supernatant was decanted and put aside. Fifty milliliters of 90% ethanol was added to the residue and centrifuged again after mixing well. This procedure was repeated three times. Ethanol was removed from the clear supernatant by rotary evaporation at a temperature below 30° C. Acidic materials were extracted from the residue by the methods used for extraction of gibberellins from barley. They were charged on a Sephadex LH-20 column (25 × 1.7 cm) and eluted with acetone. The eluate (85–160 ml) was collected. From the eluate, the fraction corresponding to GA_3 was separated and purified by silicic acid partition chromatography (8 g of silicic acid and a column with a 1.5-cm diameter were used) and preparative thin-layer chromatography, as described.

Bioassay of Gibberellins

With Dwarf Rice Seedlings. The biological activity of each fraction extracted and purified from germinating barley was examined by the microdrop method of Murakami (21) with dwarf rice seedlings (var. Tan-ginbozu).

With Deembryonated Barley Endosperm. Betzes barley was dehusked by soaking in 50% sulfuric acid for 3 hr according to the method of Pollock et al (31) and air dried for two days. The dehusked barley was cut in half with a razor blade to separate the endosperm half from the embryo. The biological activity of each fraction extracted and purified from germinating barley was also assayed with the deembryonated barley endosperm according to the method described previously (38).

Methylation and Trimethylsilylation of Purified Fractions

Ethyl ether was added to the purified gibberellin fractions, and diazomethane generated by the reaction of *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosamide and KOH was bubbled through the ethyl ether solution. After methylation, the solvent was removed in vacuo. The methylated (Me) samples were trimethylsilylated (TMS) with a reagent (200 μl) consisting of dry pyridine, 1,1,1,3,3,3-hexamethyldisilazane, and trifluoroacetic anhydride (10:9:1, v/v).

Combined GC-MS

GC-MS was performed with a JMS-D 100 mass spectrometer (JEOL Ltd., Japan) connected to a JGC 20 K gas chromatograph (JEOL Ltd., Japan). A glass column (1 m × 3 mm in diameter) packed with 2% OV-1 on Chromosorb W (AW-DMCS) 80/100 mesh was used with helium as the carrier gas at 1.2 kg/cm^2. Column and injection temperatures were 200 and 250° C, respectively. The ionizing voltage was 25 eV and the accelerating voltage was 3 kV.

Quantitative Analysis of Gibberellins

Methylated and trimethylsilylated samples were analyzed by mass fragmentography (8) by the multi-ion detection method. A peak detector (MS-PD 1, JEOL Ltd., Japan) was connected to the GC-MS. Conditions for gas chromatography and the ionization voltage were the same as those described above. Ions at a mass to charge ratio (m/z) of 506 (M^+ of GA_1-Me-TMS) and at m/z 504 (M^+ of GA_3-Me-TMS) were monitored for determination of GA_1 and GA_3 content, respectively. Standard calibration curves were set up using known amounts of authentic GA_1-Me-TMS or GA_3-Me-TMS. No correction was made for losses of gibberellins during the assay.

RESULTS AND DISCUSSION

Extraction and Purification of Endogenous Gibberellins

Several reports on endogenous gibberellins in germinating barley have been made, but only three fractions (GA_1, GA_3, and GA_7) have actually been determined. In preliminary experiments involving thin-layer chromatography and barley endosperm bioassay, only that fraction corresponding to GA_1/GA_3 exhibited the biological activity attributable to gibberellins. For this reason, the fractions corresponding to GA_1, GA_3, GA_4, and GA_7 were

purified. Ethanol was used for the extraction of gibberellins from germinating barley. Gibberellins were subsequently extracted from the ethanol extract with ethyl acetate under acidic conditions. Sephadex LH-20 column chromatography with acetone elution was effective in eliminating many impurities before the elution of GA_1, GA_3, GA_4, and GA_7. GA_1 was eluted at a ratio of elution volume to total bed volume (Ve/Vt) of 1.20–1.65, GA_3 at Ve/Vt = 1.37–1.93, and GA_4/GA_7 at Ve/Vt = 1.01–1.27. Therefore, the eluate in the range from Ve/Vt = 0.95 to Ve/Vt = 2.08, containing all of these gibberellins, was collected. After elution of the acidic materials of germinating barley, the Sephadex LH-20 column was pale yellow, a result of the absorption of impurities. The column

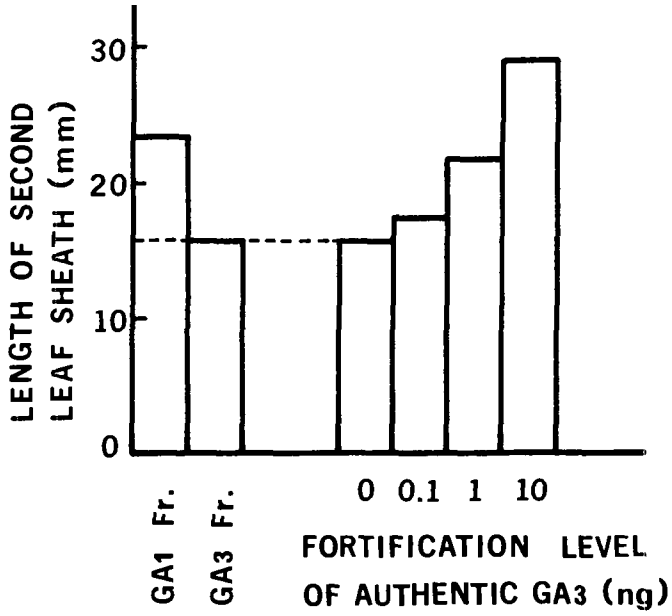

Fig. 1. Effects of the fractions (Fr.) corresponding to GA_1 and GA_3 isolated and purified from germinating barley on the growth of dwarf rice seedlings (var. Tan-ginbozu).

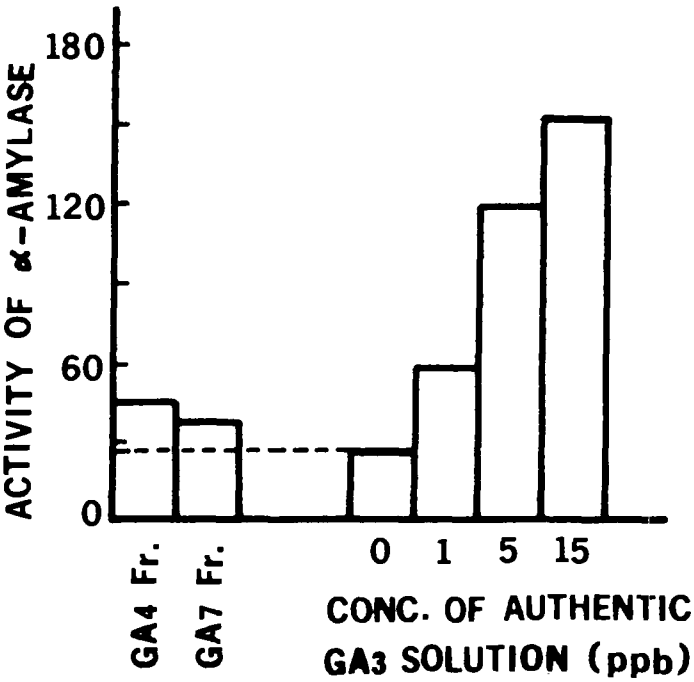

Fig. 2. Effects of the fractions (Fr.) corresponding to GA_4 and GA_7 isolated and purified from germinating barley on α-amylase formation in deembryonated endosperm.

was regenerated by elution with acetone after the absorbed impurities were washed out with methanol.

Most of the colored impurities were eluted with 90% *n*-hexane, using silicic acid partition column chromatography. The experiments with authentic gibberellins showed that GA_4/GA_7 was eluted with 85–80% *n*-hexane, GA_3 with 55–30% *n*-hexane, and GA_1 with 40–20% *n*-hexane. Accordingly, a 90–60% *n*-hexane fraction was collected as the fraction corresponding to GA_4/GA_7 and a 60–20% *n*-hexane fraction as the fraction corresponding to GA_1/GA_3.

Although GA_1 was not separated from GA_3 by thin-layer chromatography on the silica gel plate, it was possible to separate these fractions on a kieselguhr plate (GA_1 Rf = 0.72, GA_3 Rf = 0.59). However, the GA_3 fraction was contaminated by small amounts of GA_1 because of tailing. When silica gel was used as the support, GA_4/GA_7 was well separated from impurities by development with chloroform:acetic acid (95:5), but GA_4 and GA_7 were not separated. GA_4 was separated from GA_7 by development with ethyl acetate (GA_4 Rf = 0.66, GA_7 Rf = 0.58).

Biological Activity of Endogenous Gibberellins

Each gibberellin fraction purified from Fuji Nijo II barley germinated for two days was subjected to bioassay with dwarf rice seedlings. Results for the fractions corresponding to GA_1 and GA_3 are shown in Fig. 1. The fraction corresponding to GA_1 clearly elongated the leaf sheaths of the dwarf rice seedlings more than the control did, but the fraction corresponding to GA_3 did not. The fractions corresponding to GA_4 and GA_7 did not affect growth of the dwarf rice seedlings. When the barley endosperm bioassay method was used, a biological activity of the fraction corresponding to GA_3 was observed. Results for the fractions corresponding to GA_4 and GA_7 are shown in Fig. 2. Both fractions only minimally induced α-amylase formation in deembryonated barley endosperm; the activity of the fraction corresponding to GA_4 was slightly stronger than that of the fraction corresponding to GA_7.

Gibberellin fractions extracted and purified from Betzes barley germinated for two days showed the same results, which indicate that the dominant gibberellin in germinating barley is GA_1 rather than GA_3, GA_4, or GA_7.

Identification of Endogenous Gibberellins by GC-MS

After methylation and trimethylsilylation, each gibberellin fraction purified from Fuji Nijo II barley germinated for two days was subjected to GC-MS analysis. The mass spectrum of the fraction corresponding to GA_1 at the same retention time as authentic GA_1-Me-TMS is shown in Fig. 3, along with the mass spectrum of an authentic specimen. On the mass spectrum of authentic GA_1-Me-TMS, a strong molecular ion peak at m/z 506 and several fragment ions at m/z 491, 447, 377, 313, and others were observed. On the mass spectrum of the fraction corresponding to GA_1 from germinating barley, a strong ion peak was similarly observed at m/z 506 and other fragment ions at the same mass numbers as those of the authentic specimen. The molecular ion at m/z 506 and the fragment ion at m/z 491 were selected as characteristic ions of GA_1-Me-TMS, and these two ions were continuously monitored by mass fragmentography. Mass fragmentograms and the relative ratios of peak areas of m/z 506 and m/z 491 are shown in Fig. 4 and Table I, respectively.

These mass fragmentograms indicated that the authentic GA_1 sample contained an impurity (about 10%; dihydro GA_3 was suspected).[2] The retention time of the impurity was slightly shorter than that of GA_1. The peak of the mass fragmentogram of the fraction corresponding to GA_1 from germinating barley showed the same retention time as did authentic GA_1-Me-TMS. Also, the fraction corresponding to GA_1 was identical to that of authentic GA_1 in the relative ratios of the peak areas of m/z 506 and m/z 491.

[2]Prof. Takahashi, personal communication.

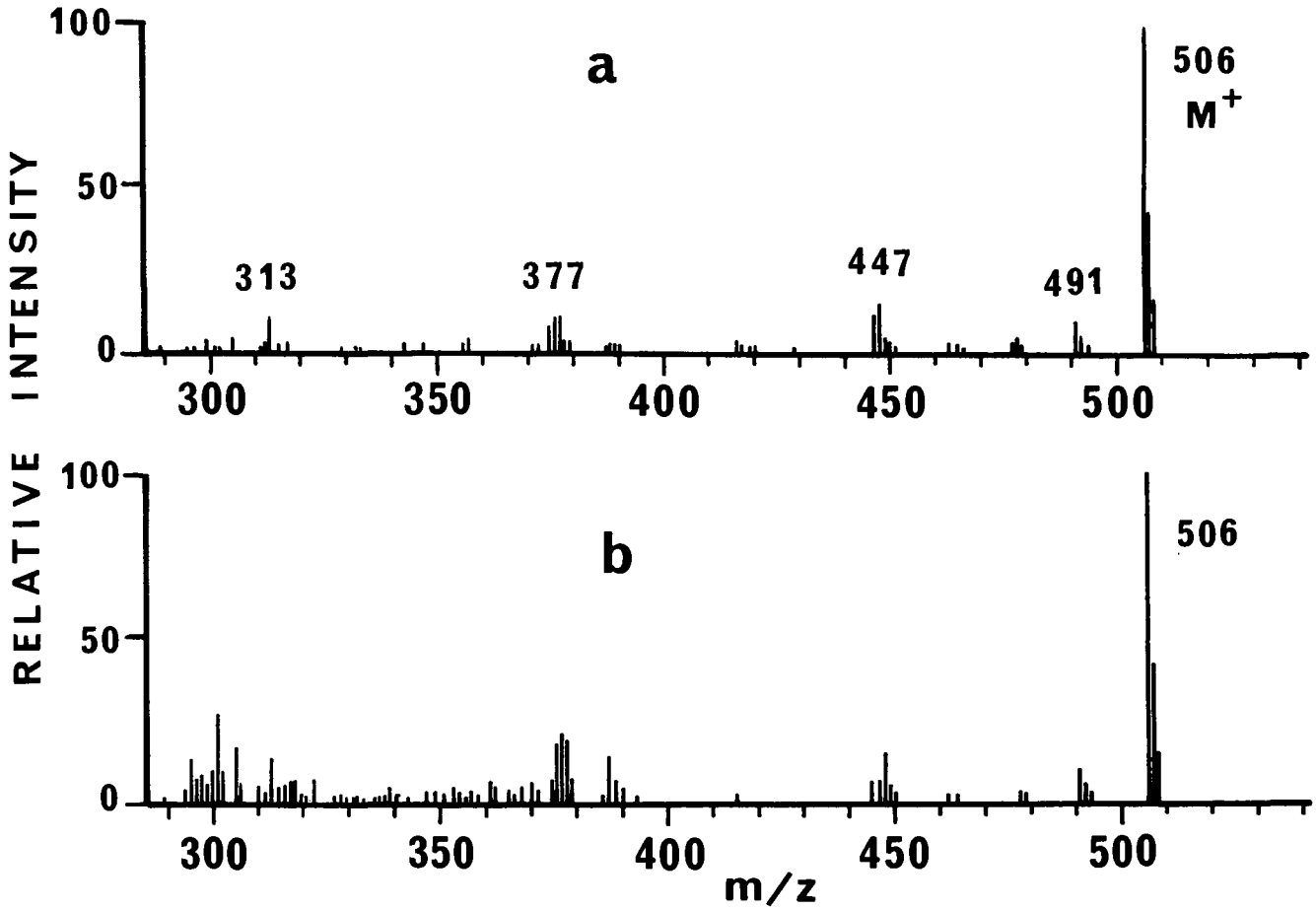

Fig. 3. Gas chromatography-mass spectrometry of the methylated trimethylsilylated (Me-TMS) derivatives of the fraction corresponding to GA_1 isolated and purified from germinating barley. **a** = mass spectrum of authentic GA_1-Me-TMS, **b** = mass spectrum of the fraction corresponding to GA_1 scanned at the same retention time as the authentic specimen.

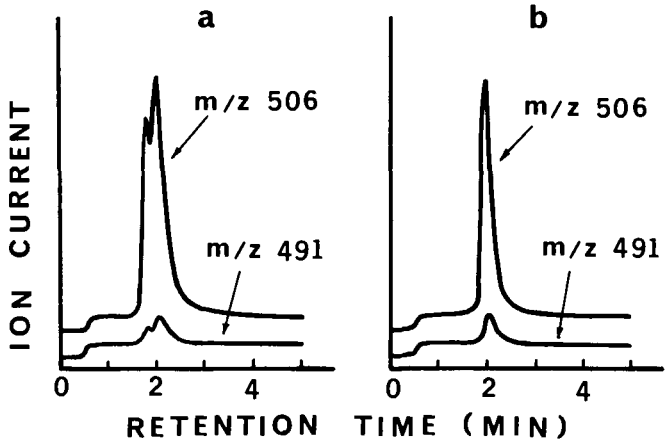

Fig. 4. Mass fragmentograms. **a** = authentic methylated trimethylsilylated (Me-TMS) GA_1, **b** = the Me-TMS derivatives of the fraction corresponding to GA_1 isolated and purified from germinating barley.

These results show that GA_1 is present in the fraction corresponding to GA_1 from germinating barley. A similar examination was made of the fraction corresponding to GA_3 from germinating barley. The mass spectrum of this fraction at the same retention time as authentic GA_3-Me-TMS is shown in Fig. 5, as is the mass spectrum of an authentic specimen of GA_3. On the mass spectrum of authentic GA_3-Me-TMS, a strong molecular ion peak was observed at m/z 504, and several fragment ions were seen at m/z 489, 475, 445, 431, 370, and others were observed. On the mass

TABLE I
Relative Ratios of Peak Areas of m/z 506 and m/z 491

	Peak Area[a]		
	m/z 506 A	m/z 491 B	Peak Ratio A/B
Authentic methylated trimethylsilylated GA_1	504	68	7.4
GA_1 fraction[b] from germinating barley	303	41	7.4

[a] Arbitrary unit.
[b] Methylated trimethylsilylated.

spectrum of the fraction corresponding to GA_3, several ions at the same mass number as the authentic specimen were observed. Many unidentified ion peaks were also observed, which presumably were contaminants or background noise. The molecular ion at m/z 504 and the fragment ion at m/z 489 were selected as characteristic ions of GA_3-Me-TMS. Mass fragmentography was used for determination of these ions. The mass fragmentogram and the relative ratios of the peak areas of m/z 504 and m/z 489 are shown in Fig. 6 and Table II, respectively. The peak at m/z 504 from the fraction corresponding to GA_3 had the same retention time as the authentic GA_3 specimen. This suggests that GA_3 was present in the fraction corresponding to GA_3 from germinating barley. The relative ratios of the peak areas of m/z 504 and m/z 489 did not correspond to those for authentic GA_3-Me-TMS, indicating that the amount of GA_3 in the fraction corresponding to GA_3 from

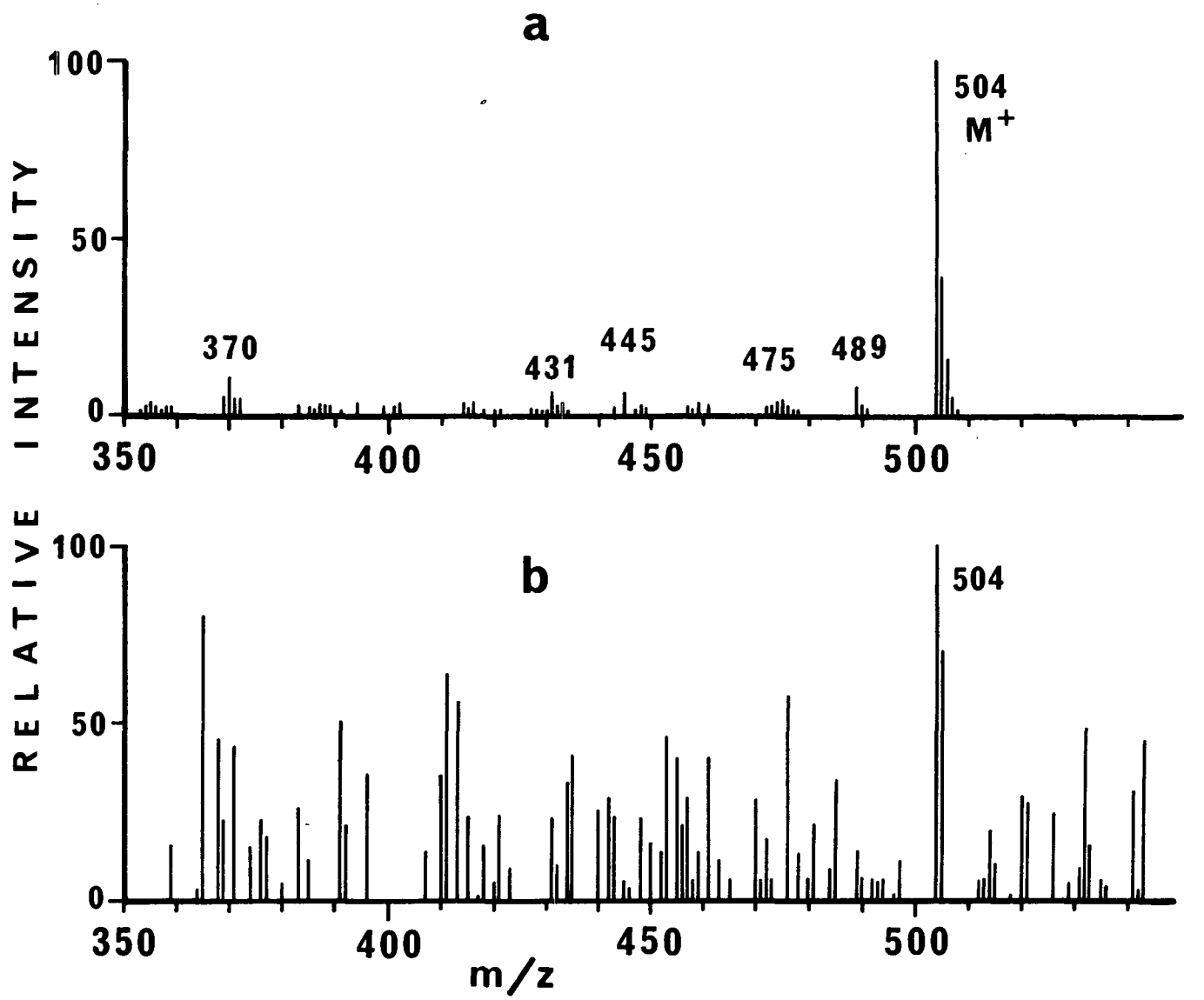

Fig. 5. Gas chromatography-mass spectrometry of the methylated trimethylsilylated (Ms-TMS) derivatives of the fraction corresponding to GA_3 isolated and purified from germinating barley. **a** = mass spectrum of authentic GA_3-Me-TMS, **b** = mass spectrum of the fraction corresponding to GA_3 scanned at the same retention time as the authentic specimen.

TABLE II
Relative Ratios of Peak Areas of m/z 504 and m/z 489

	Peak Area[a]		
	m/z 504 A	m/z 489 B	Peak Ratio A/B
Authentic methylated trimethylsilylated GA_3	737	125	5.9
GA_3 fraction[b] from germinating barley	141	61	2.3

[a] Arbitrary unit.
[b] Methylated trimethylsilylated.

germinating barley was very low and that background noise or impurities altered the peak areas on the mass fragmentograms of the fraction corresponding to GA_3.

A similar examination of the fractions corresponding to GA_4 and GA_7 purified from germinating Fuji Nijo II barley was also performed, but GA_4 and GA_7 were not identified. Betzes barley gave the same results.

Several studies have been performed to determine endogenous gibberellins in barley kernels. In immature grain, Jones et al (11) identified GA_3 and Radley (32) reported results indicating the presence of GA_4 and GA_7. The existence of gibberellins in matured barley kernels has not been reported. Using paper and thin-layer chromatography and bioassay comparison, Radley (33) reported that when barley kernels germinate, both GA_1 and GA_3 appear to be produced in the embryo, with GA_1 predominating. In contrast, Cohen and Paleg (5) and Groat and Briggs (6) examined the endogenous gibberellins in germinating barley by paper chromatography and bioassay and showed that GA_3 appeared to be the main gibberellin. Lazer et al (18,19) determined the endogenous GA_3 level in five-day green malt by an isotopic dilution procedure with paper chromatography, and Murphy and Briggs (22) identified GA_3 and GA_7 and determined the GA_3 level in germinating barley by isotopic dilution procedures with thin-layer chromatography.

The method used in this study, GC-MS, can identify compounds with greater certainty than other methods such as paper chromatography and thin-layer chromatography. In conjunction with the results of the bioassay, the corresponding retention times and mass spectra of the gibberellin fractions purified from

germinating barley and of authentic gibberellins suggest that the dominant gibberellin in germinating barley is GA_1 and that GA_3 is a minor gibberellin.

Method of Determination of GA_1 and GA_3 Levels in Germinating Barley or Malt

As shown in Figs. 3 and 5, intensities of the molecular ions of the Me-TMS derivatives of GA_1 and GA_3 are very strong, and intensities of fragment ions other than the ion at m/z 73—$Si(CH_3)_3^+$—are low. These mass spectra are characteristic of the Me-TMS derivatives of 13-hydroxy gibberellins (2). If the mass spectrometer is set to alternately bring selected ions such as m/z 504 and m/z 506 into focus on the collector slit of the detector, using the electrical switching device for accelerating voltage and the electrostatic field, only these ions are continuously detected with high sensitivity and specificity. This technique is referred to as mass fragmentography and the chromatogram obtained is a mass fragmentogram (8,36). Mass fragmentography is a valuable method for identification and determination of certain compounds on gas chromatographs below levels detectable with a flame ionization detector. Figure 7 shows a mass fragmentogram and a gas chromatogram obtained by monitoring the ion at m/z 504 and the total ion current of the Me-TMS derivatives of the fraction corresponding to GA_3 (extracted and purified from malt prepared by the addition of authentic GA_3 during malting). GA_3-Me-TMS is not clear on the gas chromatogram; however, it can be clearly seen on the mass fragmentogram at the same retention time as that of authentic GA_3-Me-TMS. The GA_3 level in the sample can be determined from the peak height or the peak area.

To test the accuracy of the method, a recovery test of authentic GA_3 added to malt was made, by the same method used for experiments on commercial malt, and the reproducibility of the method was examined by measurement of the residual GA_3 in a malt prepared by GA_3 treatment during malting. Results are shown in Table III. The recovery rate of authentic GA_3 added to malt through the preparative thin-layer chromatography step was high, and the reproducibility of the method was good.

Several methods for the determination of gibberellins have been developed. These include fluorometric procedures (9,13,35), the isotopic dilution method (18,19,22), gas chromatography (4,10), and bioassay with dwarf rice seedlings (21), deembryonated barley endosperm (1,12,26,38), dwarf peas (7), and cucumber seeds (16). Among these methods, the bioassay method, which depends on the biological activity of gibberellins, is most selective, but the bioassay results are not consistent. Fluorometric procedures lack specificity, and the isotopic dilution method is time-consuming and impractical. The sensitivity of a gas chromatograph equipped with flame ionization detector is inadequate. The GC-MS method used

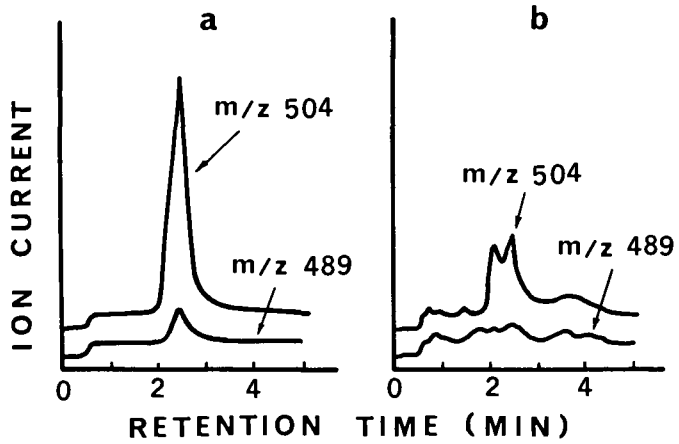

Fig. 6. Mass fragmentograms. **a** = authentic methylated trimethylsilylated (Me-TMS) GA_3, **b** = the Me-TMS derivatives of the fraction corresponding to GA_3 isolated and purified from germinating barley.

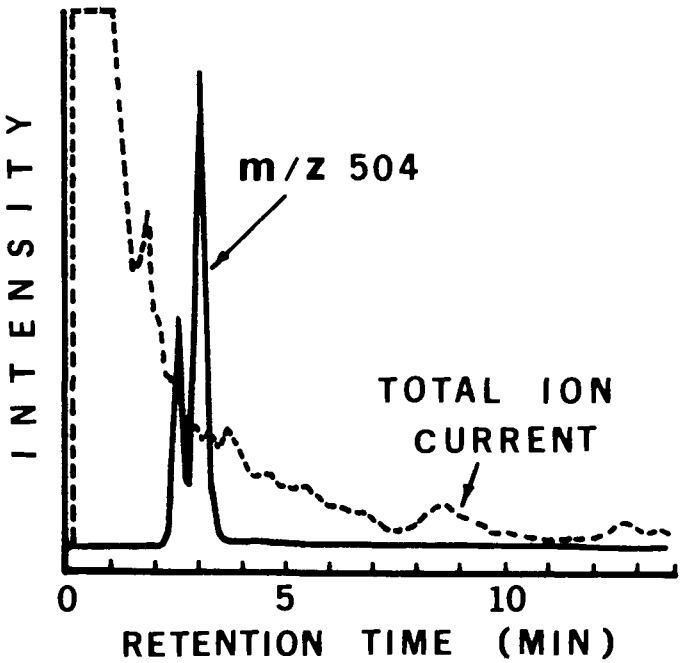

Fig. 7. Mass fragmentogram (m/z 504) and gas chromatogram (total ion current) of the methylated trimethylsilylated derivatives of the fraction corresponding to GA_3 isolated and purified from malt prepared by the addition of authentic GA_3 during malting.

TABLE III
Results of Tests of Accuracy and Reproducibility of Determination Method by Mass Fragmentography

Accuracy		Reproducibility	
Authentic GA_3	Recovery (%)	Residual GA_3[a]	Contents (μg/kg)[b]
Milligrams added to malt[c]		In experiment	
0.002	91.6	1	42.4
0.02	94.8	2	41.5
0.2		3	42.4
		4	45.2
		5	43.2
		Mean	42.9
		SD	1.4
		(SD/mean)100	3.3

[a] In GA_3-treated malt.
[b] Dry basis.
[c] 50 g of malt germinated without GA_3 treatment.

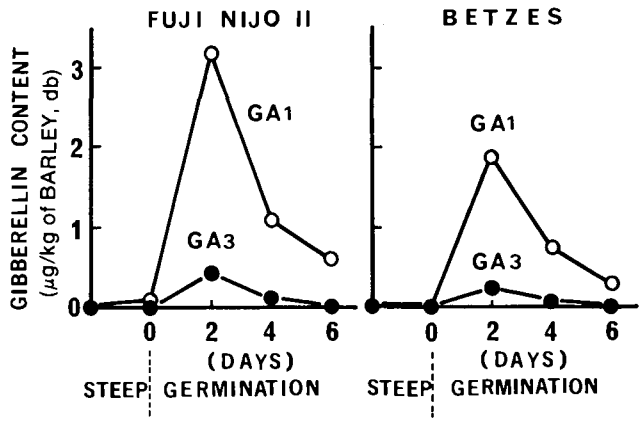

Fig. 8. Changes in the levels of endogenous GA_1 and GA_3 in germinating barley during malting.

in this study made possible the identification of certain compounds with good accuracy from the mass spectrum and retention time. The presence and levels of compounds in partially purified material can also be determined with higher sensitivity by mass fragmentography with a peak detector connected to the GC-MS. Although many studies have analyzed gibberellins with GC-MS since MacMillan's study (25) and several reports have been made on the identification and/or quantitative determination of gibberellins by mass fragmentography (3,14,15), no such reports exist on the determination of gibberellins in the malting and brewing field.

Changes in the Levels of Endogenous GA_1 and GA_3 in Germinating Barley During Malting

Changes in the levels of endogenous GA_1 and GA_3 in germinating barley under malting conditions were determined by mass fragmentography. The results are shown in Fig. 8. Before steeping, no gibberellin was detected. At the end of steeping, traces of GA_1 and GA_3 were observed. GA_1 and GA_3 contents in germinating barley reached their maximum concentrations on the second day and decreased on the fourth and sixth days of germination. Levels of GA_1 were higher than those of GA_3 throughout the germination period. Fuji Nijo II and Betzes barley gave the same results. The maximum endogenous gibberellin contents observed on the second day of germination agreed with the results of a previous article (38) and with the results of Groat and Briggs (6) and of Brooks and Martin (1). Although the levels of endogenous gibberellins (GA_1 plus GA_3) obtained in this experiment were lower by approximately one order than the levels determined by the isotopic dilution method (22) and by bioassay using induction of acid phosphatase activity in barley endosperm (1), they agreed with those of Radley (33), Coohen and Paleg (5), and the previous article (38), in which these levels were estimated by bioassay using barley endosperm.

To determine the distribution of endogenous gibberellin between the rootlets and kernel, the rootlets of Fuji Nijo II barley germinated for two days were separated by rubbing the grain on a sieve after lyophilization. GA_1 and GA_3 contents in both parts were determined by mass fragmentography. Of the GA_1, 95.7% was in the kernel and 4.3% in the rootlets; 100% of the GA_3 was in the kernel. Thus, the distribution of gibberellin in the rootlets of germinating barley was insignificant, and the value obtained from whole grain, including the rootlets, can be regarded as that of the barley kernel itself.

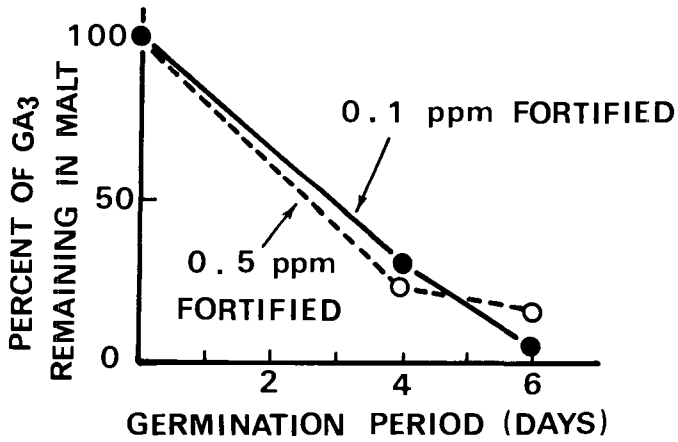

Fig. 9. Reduction in GA_3 added to steeped barley during germination.

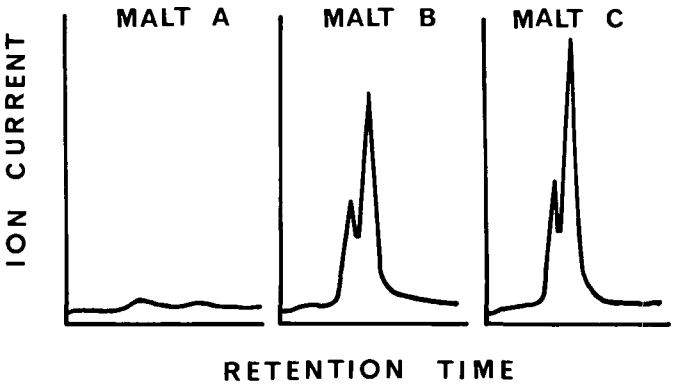

Fig. 10. Mass fragmentograms of the methylated trimethylsilylated derivatives of the fractions corresponding to GA_3 isolated and purified from three commercial malts (m/z 504). Malt A, B, and C contained 1.8, 32.1, and 36.2 μg of GA_3 per kilogram of malt, dry basis, respectively.

Behavior of Authentic GA_3 Added to Barley During Malting

Authentic GA_3 was added to steeped barley (New Golden, Japanese two-row barley cultivated in Yamanashi Prefecture, 1978 crop) at the rates of 0.1 and 0.5 mg/kg of barley, as is, and the barley samples were germinated in a pilot malting plant. The germinated barley was dried in a kiln on the fourth and sixth days of germination, and the levels of residual GA_3 in the resultant malts were determined by mass fragmentography. The results are shown in Fig. 9. The residual GA_3 in the malts decreased significantly during germination at nearly identical rates although the amounts of GA_3 added to the steeped barley were different. Brooks and Martin (1) also reported similar results. The metabolism of gibberellins in barley is not clearly understood, but the reduction in GA_3 added to barley may have been the result of its conversion to other compounds, such as GA_8 (23,27,34) or glycosides (6,23,28), which exhibit different mobility patterns at the purification step. As shown in Fig. 7, the mass fragmentogram of the fraction corresponding to GA_3 extracted and purified from malt prepared by addition of authentic GA_3 showed changes in the chemical structure of authentic GA_3 that occurred during germination of barley and/or purification. The small peak indicates a shorter retention time; however, the mass spectrum of this peak was almost the same as that of the main peak.

The pattern of the mass fragmentogram shown in Fig. 7 is characteristic of the fraction corresponding to GA_3 purified from malt prepared by GA_3 treatment during malting. Therefore, from the mass fragmentogram of this fraction, one can determine whether or not GA_3 was added to barley during malting.

The distribution of residual GA_3 between rootlets and kernel was also examined; results were similar to those obtained for endogenous gibberellins.

Determination of Residual GA_3 in Commercial Malts

Residual GA_3 in commercial malts was determined by mass fragmentography. Mass fragmentograms for three of these malts, obtained by monitoring the ion at m/z 504, are shown in Fig. 10. The facts that the dominant gibberellin in germinating barley is not GA_3 and that the fraction corresponding to GA_3 purified from GA_3-treated malt gives a characteristic mass fragmentogram made possible the easy identification of malts prepared by GA_3 treatment during malting. The level of residual GA_3 in the eight commercial malts varied from 1.8 to 36.2 μg/kg.

ACKNOWLEDGMENTS

I wish to thank N. Takahashi and N. Murofushi of the University of Tokyo for the kind gift of authentic GA_1 and for their helpful advice and also the management of Kirin Brewery Co., Ltd. for permission to publish this work. I am grateful for the continuous encouragement of Y. Kuroiwa, Director of the Research Laboratories, and Y. Horie, the former director of the Research Laboratories, and am most appreciative of the skillful assistance of M. Takahashi.

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[Received June 17, 1981]