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Effect of the Growth Stage and Cultivar on Policosanol Profiles of Barley Sprouts and Their Adenosine 5′-Monophosphate-Activated Protein Kinase Activation

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ABSTRACT: Adenosine 5′-monophosphate-activated protein kinase (AMPK) is an intracellular sensor that can regulate glucose levels within the cell. For this reason, it is well-known to be a target for drugs against diabetes and obesity. AMPK was activated significantly by the hexane extract of barley sprouts. This AMPK activation emerges across the growth stages of the sprout, becoming most significant (3 times above the initial stages) 10 days after sprouting. After this time, the activation decreased between 13 and 20 days post-sprouting. Analysis of the hexane extracts by gas chromatography−mass spectrometry showed that the amounts of policosanols (PCs, which are linear, primary aliphatic alcohols with 20−30 carbons) in the plant dramatically increased between 5 days (109.7 mg/100 g) and 10 days (343.7 mg/100 g) post-sprouting and then levels fell back down, reaching 76.4 mg/100 g at 20 days post-sprouting. This trend is consistent with PCs being the active ingredient in the barley plants. We validate this by showing that hexacosanol is an activator of AMPK. The richest cultivar for PCs was found to be the Daejin cultivar. Cultivars had a significant effect on the total PC content (113.2−183.5 mg/100 g) within the plant up to 5 days post-sprouting. However this dependence upon the cultivar was not so apparent at peak stages of PC production (10 days post-sprouting). The most abundant PC in barley sprout, hexacosanol, contributed 62−80% of the total PC content at every stage. These results are valuable to determine the optimal times of harvest to obtain the highest yield of PCs.

KEYWORDS: *Policosanol, hexacosanol, barley sprout, AMPK*

INTRODUCTION

Plants constantly synthesize secondary metabolites to adapt to their environment and enable their survival and well-being. These dynamic metabolites and their health benefits can be transferred to the plant's consumers. Research into these compounds continues to yield useful and interesting lead compounds to promote human health. Barley is one of the world's most important crops. This is because its grain is not only an excellent source of energy but also widely used in processed food stuffs because of its unique flavor and physiological benefits.[1] Barley sprouts have received much attention in recent years as a functional food in many countries, especially in Japan and Korea. It has been reported that barley leaves possess beneficial properties, such as antioxidant, hypolipidemic, antidepressant, and antidiabetic effects.[2,3] This diverse range of health benefits is probably due to the wide range of secondary metabolites contained within barley. For instance, Ferreres et al. reported that barley contains 28 water-souble phenolated glycones, whose most abundant component is isoorientin-7-*O*-glycoside (lutonarin).[4] Recently, it was found that saponarin and lutonarin are responsible for the potent antioxidant effects of young green barley leaves.[2,5]

Adenosine 5′-monophosphate-activated protein kinase (AMPK) plays a central role in metabolism by triggering adenosine 5′-triphosphate (ATP)-generating catabolic pathways.[6] AMPK is one of the best known diabetes-related signaling protein targets. This is because AMPK activation, which occurs when the enzyme is phosphorylated, leads to translocation of glucose transporter 4 (Glut4) from the cytosol to the plasma membrane, allowing glucose entry into cells and regulating glucose levels. AMPK activation also blocks ATP-consuming anabolic pathways, such as fatty acid synthesis, cholesterol synthesis, and protein synthesis. Thus, AMPK is believed to be a target for diabetes and obesity.[7−9]

Policosanol (PC) is a mixture of saturated linear, long-chain (C_{20}−C_{34}) primary alcohols derived from the waxes of plants. PCs are well-known to lower blood cholesterol and prevent

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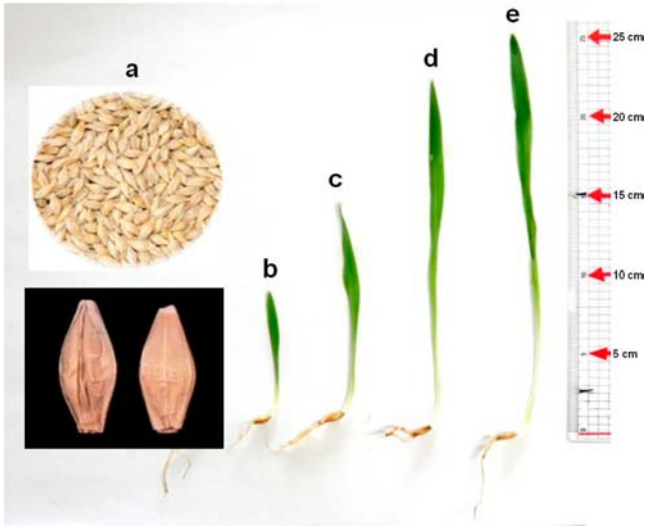

Figure 1. BSs and appearances of sprouts after germination: (a) BSs, (b) 5 days, (c) 8 days, (d) 10 days, and (e) 13 days.

low-density lipoprotein (LDL) oxidation and platelet aggregation.[10,11] These compounds are also believed to be effective in the prevention of atherosclerosis and thromboembolic disorders. These effects have been tentatively linked to inhibition of sterol biosynthesis possibly by the regulation of the activity of HMGCoA reductase mediated by AMP-dependent kinase AMPK.[12] With this in mind, we set about extracting the hydrophobic components of barley leaves to find AMP-activated protein kinase (AMPK) activators. Using an established cell-based assay, we show that hexane extracts of barley sprouts are able to activate AMPK, which was assayed by measuring an increase in AMPK phosphorylation. The degree of AMPK activation correlated with increases in expression of PCs at the different growth stages. Consistent with these data, hexacosanol, a key PC within barley sprouts, also activated AMPK dose-dependently.

MATERIALS AND METHODS

Chemicals and Reagents. Eicosanol (C_{20}), heneicosanol (C_{21}), docosanol (C_{22}), tricosanol (C_{23}), tetracosanol (C_{24}), hexacosanol (C_{26}), heptacosanol (C_{27}), octacosanol (C_{28}), and triacontanol (C_{30}) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), chloroform, and hexane were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Gibco BRL (Grand Island, NY). 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) and anti-β-actin were purchased from Sigma. Primary antibodies (anti-AMPK and antiphospho-AMPK) and secondary antibodies [anti-rabbit

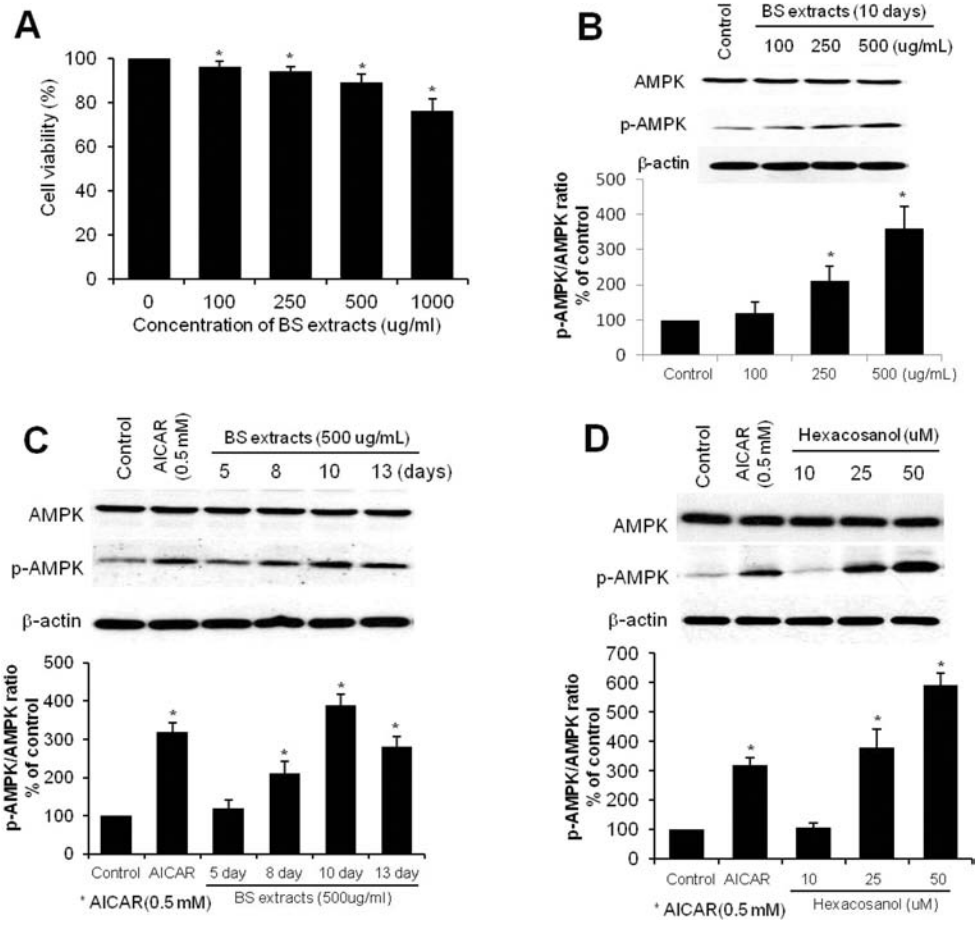

Figure 2. Effects of PC on HepG2 cells induced p-AMPK expression via activating AMPK. (A) Growth inhibition of HepG2 cells after treatment with BS extracts. Cell viability of HepG2 cells measured by the MTT assay. (B) HepG2 cells were treated with BS extracts from different growth stages for 24 h, and then p-AMPK expression was measured by western blot analysis. β-Actin was used as an internal control. (C) HepG2 cells were treated with variable concentrations of BS extracts. Under the same conditions, p-AMPK expression was measured by western blot analysis. Data represent the mean ± standard deviation of three independent experiments. (*) $p < 0.05$ when compared to corresponding control cells.

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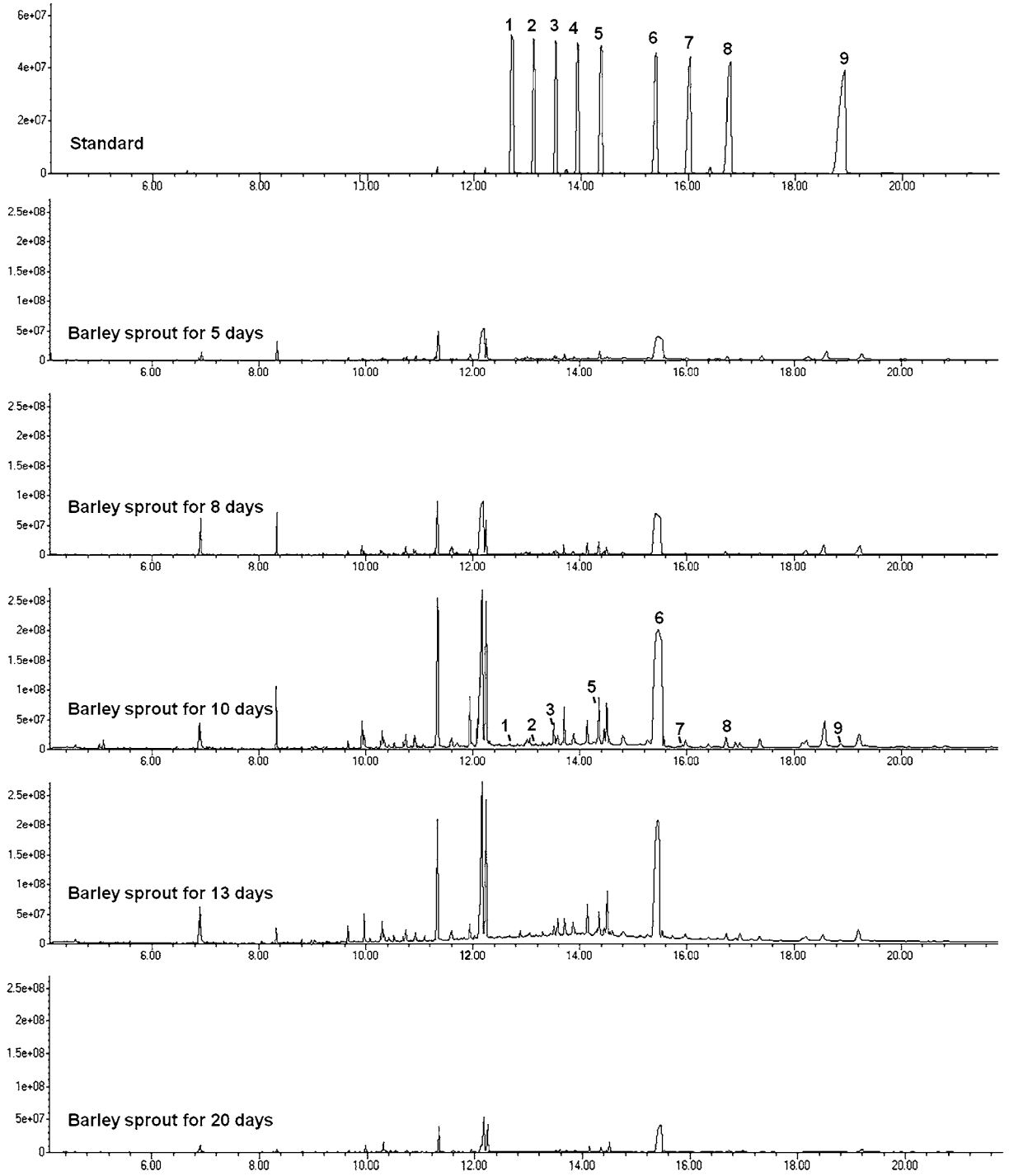

Figure 3. TIC generated using GC with a single quadrupole mass system for authentic PC−TMS derivatives. Peaks: 1, C_{20}; 2, C_{21}; 3, C_{22}; 4, C_{23}; 5, C_{24}; 6, C_{26}; 7, C_{27}; 8, C_{28}; and 9, C_{30}. TMS-derivatized extracts of barley sprouts (extract of 5 days, extract of 8 days, extract of 10 days, extract of 13 days, and extract of 20 days).

immunoglobulin G−horseradish peroxidase (IgG−HRP) and anti-mouse IgG−HRP] were purchased from Santa Cruz Biotechnology.

Plant Materials. Evaluation of PCs extracted from barley sprouts derived from 10 different cultivars, including Daejin, Nakyoung, Dahyang, Saegang, Alchan, Chal, Owl, Geungang, and Daeyoun (see Table 2), was carried out. All cultivars were obtained from the National Institute of Crop Science (NICS), Miryang, Republic of Korea. Seeds (200 g) of barley (*Hordeum vulgare* L.) were imbibed in water for 1 day and germinated in the dark over 2 days. Germinated seeds were transferred onto a modified commercial soil bed (soil bulk density, 0.7−1.0 Mg/m^3; pH, 4.5−5.5; available phosphate, 450−650 mg/L; nitrogen, 800−1000 mg/kg; Punong Bed Soil, Punong, Korea). Plants were grown in a growth chamber (DS-GC 768, Dongseo Science, Republic of Korea) at 60% relative humidity (RH) and 900−1000 lux for 8 h at 22−23 °C. Barley sprout leaves were collected over a period of 20 days. The collected leaves were freeze-dried immediately after sampling and stored at −20 °C until needed. Prior to further analysis, leaves were thawed and cut into small pieces with a laboratory blade cutter. All sample masses were based on dry weight.

Preparation of Standard and Crude Samples. A total of 1 g of dried and chopped leaves was extracted (with shaking) into hexane at

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room temperature for 24 h. After filtration, the solvent was evaporated under vacuum to give the crude extract. PCs were converted to their trimethylsilyl derivatives for identification. To a chloroform solution (0.5 mL) of PC standards was added 250 μL of MSTFA. The reaction mixture was stirred at 50 °C for 15 min followed by the addition of chloroform to obtain a 1 mL sample for analysis. Each extract was also silylated in a similar way to MSTFA for gas chromatography−mass spectrometry (GC−MS) analysis using the same procedure as standard samples.[13]

GC−MS Analysis. Trimethylsilyl alcohol derivatives were analyzed by an Agilent Technologies 7890A series GC system coupled with a 5975C single quadrupole MS (Agilent Technologies, Palo Alto, CA). A HP-5MS (5% diphenyl−95% dimethylsiloxane co-polymer) capillary GC column (30 m × 0.25 μm × 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA) was used for the analysis. The oven temperature was programmed to change from 150 to 325 °C with a 4 °C/min heating rate and then maintained at 320 °C for 5 min. Helium was used as the carrier gas at a flow rate of 1.8 mL/min. The sample (1 μL) was injected into the GC by an autosampler (Agilent Technologies, Santa Clara, CA). The split ratio was 1:5. For MS detection, the electron impact (EI) ion source and transfer line temperatures were set to 200 and 280 °C, respectively, and the ionization energy was set to 70 eV. The PC composition of each sample was identified by direct comparison of the retention times to those of authentic compounds. The samples were analyzed by GC−MS in the specific ion mode and scan mode within the same run using the retention time locked method. The data collection and analysis were managed using GC−MSD Chemstation (Agilent Technologies, Santa Clara, CA). The individual PC standards were used without further purification (97% or higher purity).

Cell Culture. HepG2 cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and were grown in DMEM supplemented with 10% FBS, glutamine (2.5 mM), and antibiotics [penicillin (50 unit/mL) and streptomycin (50 g/mL)] at 37 °C in a 5% CO_2 humidified atmosphere.[14]

Cell Viability Assay. After treatment with barley seed (BS) extracts, cells were assayed for toxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded at densities of 5000 cells/well in 96-well culture plates and were treated with the compounds for 48 h. After treatment, the attached cells were incubated with MTT solution (0.5 mg/mL, 2 h) and subsequently solubilized in dimethyl sulfoxide (DMSO). The absorbance at 550 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA).

Immunoblotting. HepG2 cells were lysed in ice-cold lysis buffer [10 mM Tris-HCl (pH 7.4), 0.1 M ethylenediaminetetraacetic acid (EDTA), 10 mM NaCl, and 0.5% Triton X-100] supplemented with a protease inhibitor cocktail (Sigma P2850) and a phosphatase inhibitor cocktail (Sigma P2850). Immunoblots were visualized using Super-Signal Pico Chemiluminescent (ECL) substrate (Pierce Chemical). Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA) as a standard.[15] Lysates (40 μg) were resolved on a 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel and then transferred to nitrocellulose membranes. After blocking with Tris-buffered saline (TBS) containing 5% nonfat dry milk and 0.1% (w/v) Tween 20, membranes were probed with primary antibody (anti-AMPK, antiphospho-AMPK, or anti-β-actin), washed 3 times in TBS, then incubated with secondary antibody (anti-rabbit IgG−HRP or anti-mouse IgG−HRP), washed 3 times in TBS, and then subjected to enhanced chemiluminescence solution and autoradiography using Hyperfilm. The relative band intensities were determined using Gel-Pro Analyzer 4.0 software (Media Cybernetics).

Statistical Analysis. Statistical analysis was undertaken using the general linear model procedure (GLM) from SAS Statistical Software Institute (version 9.1; SAS, Cary, NC). All extraction runs and analyses were carried out at least in duplicate and in randomized order, with the mean values being reported. The cell experiments were performed in triplicate. Differences between the means of the sample were analyzed by the least significant differences test at a probability level of 0.05.

RESULTS AND DISCUSSION

Identification of the PCs. PCs within the hexane extract of barley sprout were analyzed by GC−MS in the scan mode because this method can be used without sample purification.[16] Figure 3 shows the total ion chromatogram (TIC) for tetramethylsilane (TMS) derivatives of authentic PCs (C_{20}−OH, C_{21}−OH, C_{22}−OH, C_{23}−OH, C_{24}−OH, C_{26}−OH, C_{27}−OH, C_{28}−OH, and C_{30}−OH) and TMS derivatives of hexane extracts of barley sprout, respectively. Figure 3 shows that authentic eicosanol, heneicosanol, docosanol, tricosanol, tetracosanol, hexacosanol, octacosanol, and triacontanol were separated under our gas chromatographic conditions with retention times of 12.68, 13.05, 13.52, 13.93, 14.37, 15.38, 16.02, 16.77, and 18.70 min, respectively. All of the mass spectra of TMS derivatives from PCs have fragments at 57 ($C_4H_9^+$) that is characteristic of hydrocarbon chains. The peaks at 75 [$HO-Si(CH_3)_2^+$] and 103 [$CH_2OSi(CH_3)_3^+$] fragments are common for TMS alcohols. Fragmentation patterns of each TMS derivative are displayed in Table 1.

Quantification of the PCs. A comprehensive profile of the constituents of the hexane extracts of barley sprouts as a function of the growth stage was established as shown in Figure 3 and Table 2. The PC content varied significantly across the growth stages as shown by the TIC in Figure 3. We undertook a quantitative analysis of each PC peak. This showed significant changes in individual PC contents at different growth stages as shown in Figure 2. For the quantitative analysis, the nature of each peak in the TIC trace was doubly verified by comparison to the retention time of the pure compound and also by GC−MS. The concentrations of the PCs were expressed as milligrams per 100 g of barley sprout samples obtained at five different growth stages (5, 8, 10, 13, and 20 days). Levels of individual PCs in sprouts increased 3-fold from 5 days (109.7 mg) to 10 days (343.7 mg) and then rapidly decreased to 20 days (76.4 mg) on the basis of the Daejin cultivar. There is a report that hexacosanol is the most abundant PC in barley leaves, and the abundance of hexacosanol is affected by both the distance from the point of emergence (POE) and growth stage.[17] However, the previous report only covered up to 8−10 days growth post-sprouting, and the decrease in

Table 1. Chromatographic and Spectral Characteristics of the PCs in Barley Sprouts

peak	t_R (min)	MS (m/z)	pseudo-molecular ion ($[M-15]^+$)	fragments (m/z)	identification
1	12.68	370.3	355.3	355 (100), 103 (21), 83 (16), 75 (50), 57 (18)	eicosanol
2	13.05	384.4	369.4	369 (100), 103 (17), 83 (12), 75 (41), 57 (16)	heneicosanol
3	13.52	398.4	383.4	383 (100), 103 (19), 83 (14), 75 (23), 57 (18)	docosanol
4	13.93	412.4	397.4	397 (100), 103 (9), 83 (7), 75 (18), 57 (9)	tricosanol
5	14.37	426.4	411.4	411 (100), 103 (8), 83 (8), 75 (24), 57 (13)	tetracosanol
6	15.38	454.5	439.4	439 (100), 103 (18), 83 (20), 75 (40), 57 (32)	hexacosanol
7	16.02	468.5	453.5	453 (100), 103 (18), 83 (10), 75 (41), 57 (26)	heptacosanol
8	16.77	482.5	467.5	467 (100), 103 (18), 83 (15), 75 (40), 57 (27)	octacosanol
9	18.70	510.5	495.5	495 (100), 103 (17), 83 (15), 75 (40), 57 (28)	triacontanol

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Table 2. Changes of PC Contents in the Sprouts of Barley Cultivars at Different Growth Times

		PC content (mg/100 g)[a]									
stage	cultivar	C_{20}[b]	C_{21}[b]	C_{22}[b]	C_{23}[b]	C_{24}[b]	C_{26}[b]	C_{27}[b]	C_{28}[b]	C_{30}[b]	total PC[b]
5 days	Daejin	1.1 ± 0.1	nd[c]	2.7 ± 0.1	nd	8.6 ± 0.8	90.6 ± 6.3	0.6 ± 0.0	5.1 ± 0.1	0.8 ± 0.0	109.7 ± 5.6
	Nakyoung	0.7 ± 0.0	nd	4.6 ± 0.2	nd	16.7 ± 1.1	132.8 ± 10.1	1.9 ± 0.1	12.1 ± 0.5	2.7 ± 0.0	171.7 ± 7.9
	Dahyang	1.7 ± 0.1	nd	4.6 ± 0.1	nd	18.6 ± 0.9	128.4 ± 6.8	1.8 ± 0.1	12.9 ± 0.4	2.7 ± 0.0	170.9 ± 7.4
	Saegang	2.5 ± 0.1	nd	7.6 ± 0.2	nd	19.4 ± 1.0	130.7 ± 9.5	2.1 ± 0.1	13.7 ± 0.5	2.8 ± 0.0	179.2 ± 10.2
	Alchan	1.8 ± 0.0	nd	6.8 ± 0.1	nd	18.0 ± 0.9	136.1 ± 9.7	2.3 ± 0.0	15.1 ± 0.6	3.1 ± 0.1	183.5 ± 10.9
	Keunal	1.6 ± 0.0	nd	6.4 ± 0.1	nd	15.3 ± 0.7	115.3 ± 11.4	1.5 ± 0.0	11.5 ± 0.4	2.7 ± 0.0	154.6 ± 8.5
	Chal	2.7 ± 0.1	nd	6.0 ± 0.1	nd	16.0 ± 0.9	123.8 ± 6.9	1.9 ± 0.1	13.0 ± 0.4	3.1 ± 0.0	166.8 ± 6.9
	Owl	1.4 ± 0.1	nd	nd	nd	8.2 ± 0.5	94.1 ± 8.8	1.0 ± 0.0	6.8 ± 0.2	1.5 ± 0.0	113.2 ± 4.6
	Geungang	1.1 ± 0.0	nd	3.2 ± 0.0	nd	8.9 ± 0.4	95.3 ± 9.6	nd	6.1 ± 0.2	1.2 ± 0.0	116.9 ± 8.1
	Daeyoun	0.7 ± 0.0	nd	3.0 ± 0.0	nd	8.6 ± 0.6	92.8 ± 5.4	nd	6.1 ± 0.2	1.3 ± 0.0	113.4 ± 6.9
8 days	Daejin	4.5 ± 0.1	nd	16.4 ± 0.5	nd	28.8 ± 1.1	192.5 ± 10.2	2.9 ± 0.08	24.0 ± 1.1	4.6 ± 0.0	274.0 ± 12.2
	Nakyoung	3.5 ± 0.0	nd	11.2 ± 0.4	nd	23.4 ± 0.9	182.7 ± 6.8	2.7 ± 0.05	21.2 ± 1.2	4.4 ± 0.1	249.5 ± 15.6
	Dahyang	3.4 ± 0.1	nd	10.0 ± 0.6	nd	28.7 ± 2.1	195.7 ± 9.2	2.9 ± 0.03	23.5 ± 2.1	4.9 ± 0.1	269.4 ± 11.2
	Saegang	3.7 ± 0.0	nd	14.8 ± 0.6	nd	27.4 ± 1.8	183.0 ± 8.5	2.6 ± 0.05	20.4 ± 0.9	3.9 ± 0.1	256.2 ± 13.6
	Alchan	3.9 ± 0.1	nd	15.0 ± 0.5	nd	27.3 ± 1.6	208.6 ± 12.3	3.3 ± 0.06	27.4 ± 2.1	5.0 ± 0.1	291.0 ± 13.8
	Keunal	4.3 ± 0.0	nd	15.5 ± 0.8	nd	28.5 ± 1.8	204.3 ± 9.7	3.4 ± 0.04	26.2 ± 1.8	5.0 ± 0.0	287.6 ± 11.7
	Chal	3.8 ± 0.0	nd	14.7 ± 0.8	nd	25.1 ± 0.9	194.5 ± 11.7	3.0 ± 0.05	24.1 ± 1.4	4.5 ± 0.1	270.1 ± 10.9
	Owl	5.2 ± 0.1	nd	9.8 ± 0.5	nd	21.6 ± 1.2	183.3 ± 9.4	2.9 ± 0.04	22.4 ± 1.5	5.0 ± 0.1	250.6 ± 9.4
	Geungang	3.5 ± 0.1	nd	13.7 ± 0.3	nd	27.0 ± 1.2	198.9 ± 6.8	3.2 ± 0.07	25.1 ± 2.1	4.3 ± 0.1	276.2 ± 8.7
	Daeyoun	4.7 ± 0.1	nd	17.8 ± 0.7	nd	31.1 ± 1.7	206.5 ± 13.5	3.3 ± 0.04	26.3 ± 1.8	4.3 ± 0.0	294.6 ± 10.5
10 days	Daejin	5.9 ± 0.2	0.5 ± 0.0	27.0 ± 1.2	nd	38.4 ± 1.3	227.7 ± 12.1	4.1 ± 0.1	34.0 ± 1.3	5.9 ± 0.1	343.7 ± 13.1
	Nakyoung	6.0 ± 0.3	0.9 ± 0.0	22.4 ± 2.1	nd	34.1 ± 2.1	227.2 ± 13.9	4.0 ± 0.3	34.7 ± 1.1	7.0 ± 0.3	335.9 ± 10.8
	Dahyang	3.0 ± 0.1	0.6 ± 0.0	12.5 ± 1.5	nd	32.2 ± 3.8	216.9 ± 10.5	3.4 ± 0.3	28.7 ± 0.9	4.8 ± 0.3	301.9 ± 18.8
	Saegang	8.1 ± 0.2	0.5 ± 0.0	28.1 ± 0.9	nd	35.2 ± 2.1	221.7 ± 8.5	4.2 ± 0.2	30.7 ± 2.1	5.4 ± 0.3	334.2 ± 17.2
	Alchan	4.3 ± 0.3	0.8 ± 0.0	19.0 ± 0.7	nd	29.3 ± 1.9	224.2 ± 9.4	3.6 ± 0.2	34.2 ± 2.6	5.7 ± 0.4	320.8 ± 12.9
	Keunal	6.6 ± 0.4	0.9 ± 0.0	22.8 ± 1.8	nd	35.9 ± 1.8	231.4 ± 11.2	4.6 ± 0.5	34.5 ± 0.9	6.2 ± 0.2	342.4 ± 13.7
	Chal	5.2 ± 0.3	0.4 ± 0.0	23.0 ± 2.2	nd	31.3 ± 1.2	219.9 ± 10.5	4.0 ± 0.2	32.8 ± 1.4	6.0 ± 0.3	322.7 ± 19.7
	Owl	4.3 ± 0.3	0.6 ± 0.0	19.7 ± 0.8	nd	31.5 ± 1.5	226.7 ± 14.1	4.4 ± 0.1	35.3 ± 1.3	6.5 ± 0.3	329.1 ± 21.7
	Geungang	4.7 ± 0.2	0.6 ± 0.0	19.5 ± 1.1	nd	31.0 ± 1.8	217.8 ± 12.3	3.9 ± 0.3	30.8 ± 0.9	5.0 ± 0.3	313.2 ± 10.8
	Daeyoun	4.9 ± 0.2	0.4 ± 0.0	22.5 ± 0.7	nd	32.8 ± 1.6	216.4 ± 11.8	3.7 ± 0.2	29.0 ± 0.8	4.2 ± 0.4	314.0 ± 14.8
13 days	Daejin	5.8 ± 0.3	0.5 ± 0.0	31.2 ± 1.4	nd	40.1 ± 3.1	223.9 ± 11.4	4.4 ± 0.2	25.7 ± 1.2	7.6 ± 0.9	339.4 ± 16.3
	Nakyoung	3.2 ± 0.1	0.2 ± 0.0	18.8 ± 2.2	nd	28.7 ± 4.5	203.3 ± 13.5	3.3 ± 0.3	29.7 ± 1.6	5.8 ± 0.5	293.2 ± 11.2
	Dahyang	2.2 ± 0.3	0.2 ± 0.0	12.2 ± 1.6	nd	27.0 ± 2.5	192.7 ± 16.4	3.0 ± 0.3	25.8 ± 2.2	4.4 ± 0.2	267.5 ± 26.1
	Saegang	4.8 ± 0.5	0.4 ± 0.0	24.8 ± 1.9	nd	29.8 ± 1.8	194.6 ± 18.5	3.4 ± 0.2	28.5 ± 1.4	5.0 ± 0.8	291.4 ± 18.5
	Alchan	3.1 ± 0.5	0.2 ± 0.0	17.1 ± 2.3	nd	25.7 ± 1.7	194.8 ± 15.7	3.2 ± 0.4	29.9 ± 0.5	5.2 ± 0.2	279.4 ± 13.5
	Keunal	4.2 ± 0.2	0.3 ± 0.0	22.5 ± 1.8	nd	26.3 ± 2.6	185.6 ± 11.2	3.0 ± 0.4	27.8 ± 1.9	4.9 ± 0.3	274.8 ± 14.9
	Chal	3.0 ± 0.2	0.2 ± 0.0	17.5 ± 2.0	nd	22.7 ± 1.1	166.8 ± 10.6	2.4 ± 0.5	24.2 ± 1.7	4.5 ± 0.3	241.4 ± 12.8
	Owl	3.8 ± 0.2	0.2 ± 0.0	16.6 ± 1.2	nd	23.8 ± 2.1	187.3 ± 14.5	3.0 ± 0.2	29.0 ± 0.8	5.1 ± 0.4	268.9 ± 16.9
	Geungang	3.3 ± 0.3	0.2 ± 0.0	13.9 ± 0.5	nd	21.3 ± 1.4	175.7 ± 10.3	2.5 ± 0.2	24.6 ± 1.4	3.6 ± 0.6	245.3 ± 16.7
	Daeyoun	3.6 ± 0.2	0.3 ± 0.0	17.0 ± 1.1	nd	23.3 ± 0.9	174.5 ± 9.4	2.6 ± 0.3	20.4 ± 2.9	2.8 ± 0.1	244.6 ± 14.9
20 days	Daejin	nd	nd	0.9 ± 0.1	nd	4.6 ± 0.1	70.6 ± 4.2	nd	nd	0.3 ± 0.0	76.4 ± 3.2
	Nakyoung	nd	nd	1.5 ± 0.2	nd	7.7 ± 0.2	105.4 ± 9.5	nd	3.1 ± 0.2	0.9 ± 0.1	118.6 ± 4.1
	Dahyang	nd	nd	1.2 ± 0.1	nd	8.6 ± 0.1	98.6 ± 4.1	nd	4.9 ± 0.2	0.9 ± 0.1	114.2 ± 5.6
	Saegang	nd	nd	0.9 ± 0.2	nd	8.4 ± 0.2	110.1 ± 5.3	nd	3.7 ± 0.2	1.1 ± 0.1	124.2 ± 7.2
	Alchan	nd	nd	1.5 ± 0.1	nd	8.0 ± 0.3	115.2 ± 5.9	nd	4.1 ± 0.2	1.1 ± 0.0	129.9 ± 7.9
	Keunal	nd	nd	1.1 ± 0.1	nd	6.3 ± 0.1	110.4 ± 4.8	nd	5.1 ± 0.1	0.6 ± 0.0	123.5 ± 7.8
	Chal	nd	nd	1.0 ± 0.1	nd	5.0 ± 0.2	101.6 ± 9.5	nd	3.2 ± 0.2	0.8 ± 0.1	111.6 ± 9.2
	Owl	nd	nd	nd	nd	3.2 ± 0.1	68.1 ± 3.1	nd	2.8 ± 0.2	0.5 ± 0.0	74.6 ± 4.5
	Geungang	nd	nd	0.8 ± 0.0	nd	3.3 ± 0.1	72.1. ± 4.2	nd	2.1 ± 0.2	nd	78.3 ± 4.9
	Daeyoun	nd	nd	0.9 ± 0.0	nd	2.6 ± 0.1	62.7 ± 3.4	nd	2.4 ± 0.2	nd	68.6 ± 6.1

[a]All values are the mean ± standard deviation of three independent experiments. [b]C_{20}, eicosanol; C_{21}, heneicosanol; C_{22}, docosanol; C_{23}, tricosanol; C_{24}, tetracosanol; C_{26}, hexacosanol; C_{27}, heptacosanol; C_{28}, octacosanol; C_{30}, triacontanol; and total PC, total PC content. [c]nd = not detected.

abundance was missed. Thus, the optimal time for harvest was unclear. Consistent with the previous report, hexacosanol was the most abundant compound, and its content was directly proportional to the growth stage until 10 days post-sprouting: 90.6 (5 days) < 192.5 (8 days) < 227.7 (10 days). However, after this point, we present new data showing that hexacosanol levels rapidly decreased and reached basal levels at 20 days post-sprouting (70.6 mg).

If barely sprouts are to be used as a functional food providing PCs, then not only the optimal harvest times but also the best cultivars must be known. We set out to establish these important facts in Table 2. For all cultivars studied, the concentration of PCs increased with the growth stage until 10 days post-sprouting. Hexacosanol was the most abundant PC in all varieties. PC contents also decreased across growth stages from the climatic stage (10 days) up to 20 days post-sprouting. The most rapid

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increase in productions during the early growth period was observed for the C_{28} PC. The content of this compound increased almost 6-fold between 5 days post-sprouting (5.1 mg) and 10 days post-sprouting (34.0 mg). In contrast, the increase in the hexacosanol concentration was 82% at 5 days post-sprouting and 66% at 10 days post-sprouting. Interestingly, although the total level of PCs at the 5 days post-sprouting stage varied depending upon cultivars used [109.7 mg (Daejin) and 183.5 mg (Alchan)], the total PC level at 10 days post-sprouting was not much different for any cultivar.

Phosphorylation of AMPK. The study began by collecting barley sprout leaves sequentially at growth stages between 5 days (around 5−8 cm) and 20 days (around 45−50 cm) after seeding and extracting them into hexane (Figure 1). The crude hexane extracts were screened and evaluated initially for their ability to activate AMPK and, subsequently, for their PC contents by GC−MS. The hexane extract used to test changes in AMPK regulation was obtained from barley sprouts of Daejin (the richest cultivar for PCs at 10 days post-sprouting). All assessments of AMPK activation were carried out according to a standard literature procedure,[18] which involves following the effect of each extract on AMPK phosphorylation in HepG2 cells.

First of all, the effects of the extract on cell viability were assessed using a colorimetric MTT-based assay, as described in the Materials and Methods. The hexane extract of barley sprouts emerged to have very low cytotoxicity up to 1 mg/mL (Figure 2A). As shown in Figure 2B, we investigated the dose-dependent effects that the hexane extract of leaves at 10 days post-sprouting had on both the expression levels of AMPK and the phophorylation of AMPK by western blot analysis in HepG2 cells. AICAR was used as a positive control to stimulate AMPK phosphorylation.[14,19] We determined the relative effectiveness of each extract at each growth stage (5, 8, 10, 13, and 20 days). The extracts at 5 days post-sprouting showed little difference relative to the control. However, statistically significant 200 and 400% increases of activation potencies were observed for extracts obtained 8 and 10 days post-sprouting, respectively. AMPK activation tailed off at 13 days post-sprouting (290%) and returned to levels similar to the control at 20 days post-sprouting.

Because a tentative link between AMPK activation and PCs had been made previously,[12,20] we hypothesized that the phosphorylation of AMPK would be affected by PCs in barley sprouts. We tested this hypothesis by first showing that an authentic sample of hexacosanol, which we shown above to be the most abundant PC in the sprouts, was able to elicit AMPK activation in a similar way to the extracts. The effects of hexacosanol (C_{26}) on both the expression levels of AMPK and AMPK phosphorylation were investigated. Dose-dependent activation of AMPK phosphorylation by pure hexacosanol was observed clearly. The activation trend agreed with previous results[12] and presents a coherent picture that hexacosanol within barley sprouts is responsible for the AMPK activation that we observe. Thus, although other PCs, particularly octacosanol, have been the focus of many studies in AMPK activation, it now appears that hexacosanol warrants more attention.

We thus clearly relate AMPK activation by barley sprout extracts to the PC levels in the plant. This, to our knowledge, is the first time such an analysis has been undertaken. We establish the link between AMPK activation and PC content using both a detailed analysis of PC expression as a function of the growth stage, which correlates with the onset, and an increase in AMPK activation shown by the extract. We also demonstrate that the most abundant PC in barley leaves is an AMPK activator.

These data clearly link barley leaves to a wide range of health benefits associated with AMPK activation. Our detailed analysis of the components as a function of cultivars and growth stages provides an excellent guide to optimize harvesting times based on maximum bulk yield and PC expression.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

PC, policosanol; AMPK, adenosine 5′-monophosphate-activated protein kinase; BS, barley seed

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