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Article

Amplification of Sulforaphane Content in Red Cabbage by Pressure and Temperature Treatments

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Red cabbage, a *Brassica* vegetable, has glucoraphanin that is hydrolyzed by myrosinase to form sulforaphane, which has received attention due to its cancer chemopreventive activity. High hydrostatic pressure (HHP) treatment (100~400 MPa) and subsequent incubation (20~80°C) were employed to amplify health beneficial sulforaphane content in red cabbage. The highest quantity of sulforaphane was 99.7 μmol/kg fresh weight from HHP treatment at 400 MPa, followed by standing at 60°C. Cytotoxicity was determined to evaluate the side effect of HHP-treated red cabbage.

Key words: *Brassica oleracea* L. var. *capitata* f. *rubra* DC., glucoraphanin, high hydrostatic pressure, red cabbage, sulforaphane

Brassica vegetables are known to possess cancer chemopreventive potency due to their unique compounds stored in the form of glucosinolates (GLSs) [Hayes *et al.*, 2008]. In intact vegetables, GLSs are stably localized in vacuoles, but rapidly hydrolyzed when in contact with myrosinase (thioglucoside glucohydrolase EC 3.2.3.1), an enzyme located in vacuole-like structures in special myrosin cells [Jones *et al.*, 2006]. Hydrolysis products include isothiocyanates (ITCs), thiocyanates, nitriles, epithionitriles, hydroxynitriles, and oxazolidine-2-thiones [Fahey *et al.*, 2001; Jones *et al.*, 2006]. Among them, ITCs are regarded to be primarily responsible for the cancer chemopreventive agents [Zhang *et al.*, 1992; Munday and Munday, 2004].

To generate heath beneficial ITCs, cell membrane disruption such as cutting, chopping, grinding, and chewing is required to bring myrosinase into contact with GLSs. During many conventional cooking, however, heat is applied to the vegetables before the tissue disruption for blanching or pasteurization, resulting in the inactivation of both myrosinase and generation of beneficial ITCs. According to Van Eylen *et al.* [2009], high hydrostatic pressure (HHP) treatment, a fast growing non-thermal technology, is a promising approach to accumulate more ITCs in *Brassica* vegetables before any heat treatment. Their study showed that the treatment of broccoli by high pressure (100-500 MPa) at moderate temperatures (20-40°C) promoted the conversion of GLSs into ITCs.

Red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra* DC.) has been mostly consumed as salad, coleslaw, and beverage. It shows cancer chemopreventive property [Uhl *et al.*, 2004] and contains glucoraphanin, sinigrin, and glucoiberin as major GLSs [Meyer and Adam, 2008]. Among them, glucoraphanin is transformed into sulforaphane, which is a very well known cancer chemopreventive ITC [Clarke *et al.*, 2008]. So far, the increase of sulforaphane content after harvest has been mainly studied in broccoli, but hardly studied in other cruciferous vegetables including red cabbage. In the present study, HHP treatment was applied to red cabbage to amplify health beneficial sulforaphane. Different pressures and incubation temperatures were evaluated for the generation of sulforaphane in red cabbage. Finally, cytotoxicity of sulforaphane was determined to evaluate side effects of red cabbage by HHP treatment.

Materials and Methods

Preparation and treatment of red cabbage. Red cabbage (*Brassica oleracea* L. var. *capitata* f. *rubra* DC.)

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was purchased from local markets in Gangneung, Korea and stored at 4°C until further use. After removing the outer leaves, 50 g of the red cabbage was cut into pieces of approximately 7×7 cm^2 and packed in a heat-sealed vacuum bag. The sample bags were placed in a high-pressure vessel of laboratory-scale with a filling volume of 2.5 L (Autoclave Engineering Systems Inc., Columbus, OH). HHP treatment was performed at four different pressures (100, 200, 300, and 400 MPa) for 10 min, not including the pressure build-up and release time. The temperature of the vessel was maintained at 25°C using an external water circulator (RW-1025G, Lab Companion, Seoul, Korea). After HHP treatment, the vacuumed sample bags were incubated at four different temperatures (20, 40, 60, and 80°C) for 1 h to induce sulforaphane formation by myrosinase. Blanching was carried out at 100°C for 3 min, and the samples were ground in a domestic grinder.

Identification and quantification of sulforaphane. Red cabbage (50 g) was extracted with 80 mL methanol at room temperature for 1 h and filtered through Whatman No.1 filter paper into a 100-mL volumetric flask. The extract was brought up to the volume of the flask with methanol. The sulforaphane of red cabbage extract was quantitatively analyzed using liquid chromatography/mass spectrometry (LC/MS) (HP-1100MSD, Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization interface in the positive mode by the modified method of Agrawal *et al.* [2006]. Separation was performed on a Waters XTerra™ C18 column (250 mm×4.6 mm, 5 μm; Milford, MA) using a solvent system of acetonitrile-water (20:80 v/v at 0 min to 60:40 v/v at 20 min). The flow rate was 0.5 mL/min and injection volume was 10 μL. Operating parameters of the electrospray ionization-mass spectrometry (ESI-MS) were optimized by direct infusion of sulforaphane standard solution in the mobile phase: positive ion mode, dry gas flow (N_2) 13 L/min, nebulizer pressure 30 psi, drying gas temperature 350°C, and capillary voltage at 4500 V. Mass spectra were acquired over the scan range *m/z* 400-700 using a 0.1 unit step size.

Cytotoxicity. To assess the cytotoxicity of the processed sample on the various cell lines, the cell proliferation assay was performed using the Cell Counting Kit (CCK-8) produced by Dojindo Laboratories (Tokyo, Japan). In brief, HCT116 cells (5×10^3 cells per well), A549 cells (1×10^4 cells per well), MCF-7 cells (2×10^4 cells per well), and Hepa1C1C7 cells (1×10^4 cells per well) were plated into the wells of 96-well plates, incubated at 37°C for 24 h, and the cells were treated with various concentrations of the processed samples. After 24 h treatment, 10 μL CCK-8 solution was added to the wells, and incubation was continued for additional 1 h, and absorbance at 450 nm was then measured by a PowerWave™ XS Microplate Spectrophotometer reader (Bio-Tek Instruments, Winooski, VT). The samples were dissolved in dimethyl sulfoxide (DMSO). The cytotoxicity (IC_{50}) was calculated as the concentration (mg/mL) at which 50% inhibition of cell proliferation is shown.

Statistics. The mean values and standard deviations of experiments were calculated from data obtained with triplicate trials. The data were analyzed by Student's *t*-test ($p<0.05$) for all analysis.

Results and Discussion

Sulforaphane is derived from glucoraphanin by myrosinase in *Brassica* vegetables including red cabbage. Because myrosinase and glucoraphanin are segregated physically in intact cell, cell membrane disruption is required to bring myrosinase into contact with glucoraphanin, giving rise to the production of sulforaphane (Fig. 1). Humans can take sulforaphane by chewing ground raw *Brassica* vegetables. However, heat applied to the vegetables during many conventional cooking, results in the inactivation of myrosinase before the generation of beneficial ITCs. In the present study, HHP treatment was employed as a physical disruption process, because it was expected to have the advantage of making leakage in membrane of red cabbage without affecting the physical appearance.

Methanol extract of red cabbage was separated in the

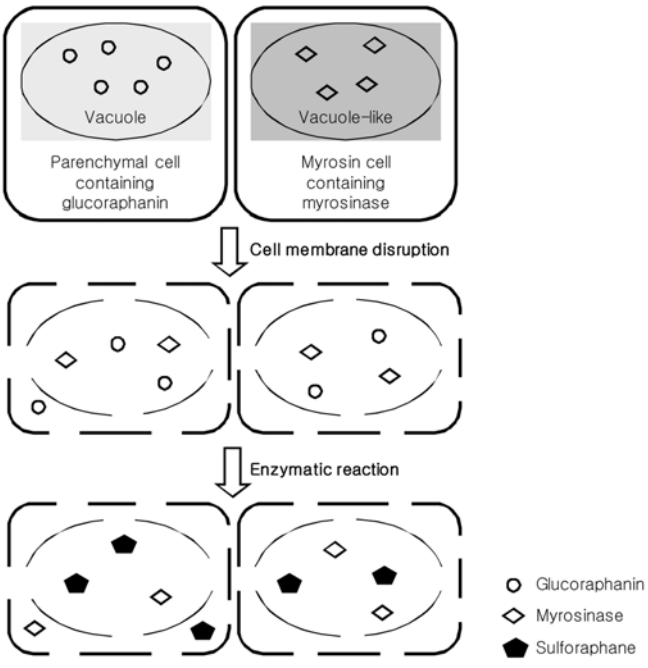

Fig. 1. Schematic diagram showing sulforaphane production from glucoraphanin and myrosinase in red cabbage.

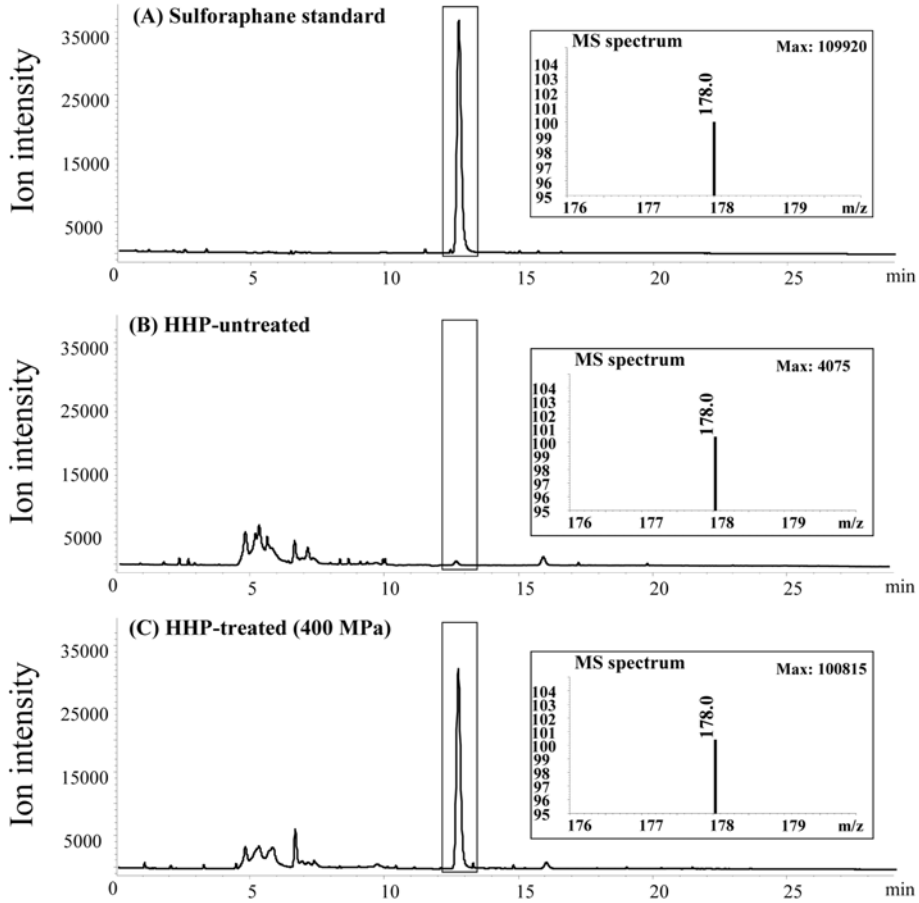

Fig. 2. LC/MS chromatogram and MS spectrum of (A) sulforaphane standard, (B) HHP-untreated, and (C) HHP-treated red cabbage.

column, and the ion intensity of effluent was detected. Sulforaphane was identified on the basis of its retention behavior compared to authentic standard and further confirmed by its *m/z* value (Fig. 2). The retention time of sulforaphane was 12.74 min. To determine the calibration curve of sulforaphane, standard solutions of sulforaphane were analyzed by LC/MS. Subsequently, the peak area (Y) of sulforaphane was measured and plotted against the concentration (X) of sulforaphane. The regression equation was Y=14814 X+21127 (n=5). A good linearity was obtained between peak areas and concentrations at the linear range of 3-50 μg/mL with the correlation coefficients of 0.9981. As expected, sulforaphane content in intact red cabbage was too low to be analyzed quantitatively (Fig. 2B). Thus, the effects of physical processings such as blanching, grinding, and HHP treatment on the sulforaphane content were examined. As in the intact red cabbage, sulforaphane content was too low to be analyzed in blanched red cabbage (data not shown). In ground red cabbage, sulforaphane could be detected by MS, but its content was too low to be analyzed (data not shown). On the other hand, the sulforaphane content showed a definite increased in HHP-treated red cabbage at 400 MPa (Fig. 2C). A clear difference in sulforaphane content was also found between other processed and HHP-treated red cabbages.

The effect of HHP treatment and incubation temperature on sulforaphane yield is shown in Fig. 3. In HHP-untreated sample, sulforaphane was scarcely detected. From the extrapolation of the calibration curve of sulforaphane, the content of sulforaphane was estimated to be 0.05 μmol/kg fresh weight at all temperature range in HHP-untreated red cabbage. HHP treatment of red cabbage increased sulforaphane yield with the increase of the applied pressure, which implied that higher pressure up to at least 400 MPa could disrupt the cell membrane of red cabbage efficiently. The most effective incubation temperature after HHP treatment was 60°C. This tendency was observed in all HHP treatments at different pressures and may be closely related to the optimum temperature and the thermal stability of endogenous myrosinase in red cabbage. Overall, the highest quantity of sulforaphane was 99.7 μmol/kg fresh weight from HHP treatment at 400 MPa followed by standing at 60°C.

Generally, glucoraphanin, one of the GLSs in *Brassica* vegetables breaks down to either sulforaphane or sulforaphane

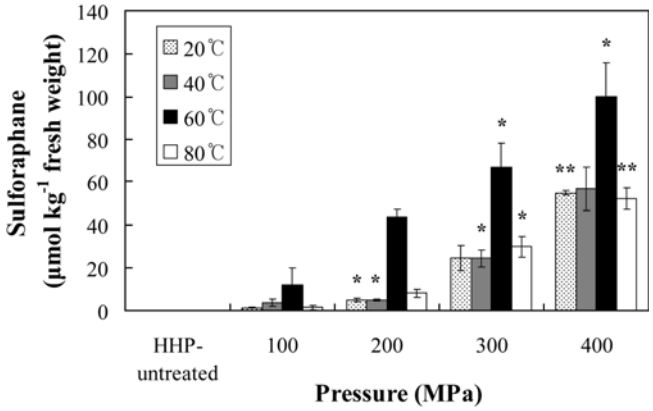

Fig. 3. The effect of high hydrostatic pressure treatment (HHP) and subsequent incubation temperature on sulforaphane yield in red cabbage. **p<0.005, *p<0.01 using Student's t-test; n=3.

nitrile depending on the conditions of hydrolysis. Sulforaphane is known to be potent chemopreventive and anticarcinogenic agent. However, although sulforaphane nitrile is the predominant hydrolysis product of glucoraphanin, it has been shown to not possess the anticarcinogenic properties of sulforaphane. Thus, the potential health benefit of *Brassica* vegetables results from not sulforaphane nitrile but via sulforaphane formation. Epithiospecifier protein (ESP), identified in some *Brassica* vegetables, hydrolyzes glucosinolate into epithionitriles rather than isothiocyanates. Heat treatment has been shown to decrease the formation of epithionitriles [Matusheski *et al.*, 2004]. Rungapamestry *et al*. [2006] reported that ESP activity siginificantly decreased at 50°C and above, resulting in lower nitrile content, and myrosinase activity was also decreased at 60°C and above. In the present study, to reduce the formantion of inactive sulforaphane nitrile, the incubation process was performed after HHP treatment with focus on the sulforaphane formation. HHP treatment followed by incubation at 60°C produced the highest quantity of sulforaphane, implying that the conditions may decrease the ESP activity but retain the myrosinase activity.

To evaluate the side effects resulting from HHP treatment of red cabbage, cytotoxicity of the extract of HHP-treated red cabbage was compared with that of HHP-untreated red cabbage using human colorectal carcinoma (HCT116), human lung carcinoma (A549), human breast adenocarcinoma (MCF-7), and mouse hepatocellular carcinoma (Hepa1C1C7) cell lines. None of the samples showed cytotoxic effect on all cell lines up to 2 mg/mL. Actually, the reaction occurring in red cabbage by HHP treatment is thought to be quite similar to that taking place during chewing of the red cabbage. It may imply that HHP-treated red cabbage is a safe food strengthening health benefit.

Some studies have reported that cooking of *Brassica* vegetables can substantially reduce or destroy isothiocyanate, and the normal cooking can inactivate myrosinase, destroy heat-labile isothiocyanates, and lose glucosinolates due to excessive heat. Although human intestinal microflora also possess myrosinase activity and are able to partially hydrolyze ingested glucosinolates, it has been reported that the activity of isothiocyanate after the consumption of cooked vegetables is 60 to 90% less than after the ingestion of raw *Brassica* vegetables [Tang *et al*., 2008]. Considering these results, our findings suggest that HHP technique is nonthermal processing and can effectively amplify the sulforaphane content in raw red cabbage. The highest yield of sulforaphane can be obtained from HHP treatment at 400 MPa followed by standing at 60°C as compared with intact, blanched or ground red cabbage. HHP processing of *Brassica* vegetables may provide a means of increasing the uptake of isothiocyanate, sulforaphane.

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