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Short communication

Characterization of benzyl isothiocyanate extracted from mashed green papaya by distillation

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ABSTRACT

The aim of this study was to extract benzyl isothiocyanate (BITC) from green papaya by distillation apparatus without using organic solvents, and to improve the stability of BITC in aqueous solution. The distillation of mashed green papaya successfully yielded BITC as a water solution with more than 80% purity with good reproducibility. The amount of BITC in the distilled water gradually decreased during its storage at 4 °C, whereas it was not significantly changed at −20 °C for a few months. Moreover, the addition of L-cysteine ameliorated the BITC decomposition by the 4 °C-storage, but not affected by *N*-acetyl-cysteine and glutathione. These results suggested that the combination of BITC extraction by distillation and cysteine supplementation as well as frozen storage might be a useful method for the preparation and storage of the safer grade of BITC-containing extract.

1. Introduction

Isothiocyanates (ITCs) are organic sulfur compounds that are mainly contained in cruciferous vegetables. ITCs have been reported to have human health-promoting properties, such as antioxidative, anti-inflammatory, and anti-cancer effects (Mi, Di Pasqua, & Chung, 2011; Nakamura, Abe-Kanoh, & Nakamura, 2018). Although ITCs have these effects, ITCs are easily decomposed by the addition of a hydroxyl ion to the ITC group under a neutral or alkaline condition (Kawakishi & Namiki, 1969; Pecháček, Velíšek, & Hrabcová, 1997). In addition, the rearrangement of the ITCs into thiocyanates occurs during the glucosinolate metabolism or storage in aqueous solutions, resulting that the biological effectiveness of ITCs is considerably reduced. Thus, ITCs in an aqueous solution might be too unstable to utilize as food additives.

As for *in vivo* situation, ITCs can quickly conjugate with the reduced-form glutathione (GSH), the most abundant thiol compound in the cells, as well as L-cysteine and form dithiocarbamates (DTCs). The DTCs themselves are also unstable in an aqueous buffer because the conjugates can be dissociated into free ITC and thiol compounds. However, the dissociated ITC can react with a free thiol compound again, and then DTCs exist in equilibrium with the free form (Jiao et al., 1996). Thus, the stability of ITC itself is thought to ameliorate by the addition of the thiol compounds to maintain the conjugated forms. However, there are only limited reports concerning the stability of ITCs and DTCs in aqueous solutions.

Based on the chemical characteristics of the ITCs, such as hydrophobicity and instability, organic solvents have been used for the extraction of the ITCs from food materials with good yield. Fahey *et al.* has shown that 4-methylsulfinylbutyl ITC (sulforaphane) was extracted from broccoli sprouts using dimethyl sulfoxide (DMSO) and acetonitrile (Fahey, Zhang, & Talalay, 1997). Nakamura *et al.* has also reported that BITC was extracted from papaya seed and edible pulp using methanol and hexane with a substantive recovery (Nakamura et al., 2007). However, the extraction of the chemicals using organic solvents is not suitable for the preparation of food-grade products. In addition, to the best of our knowledge, there is no report showing that ITCs can be extracted as aqueous solutions.

The aim of this study is to extract BITC from green papaya, which is one of the BITC-rich plant materials, by distillation without using organic solvents, and also to improve the stability of BITC in aqueous solution. In this study, BITC was successfully extracted from mashed green papaya by distillation without using an organic solvent with the relatively high purity. Moreover, the present study demonstrated that not only frozen storage, but also supplementation of L-cysteine allows the stability of BITC in the distilled water to be maintained.

Abbreviations: AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; DMSO, dimethyl sulfoxide; DTCs, dithiocarbamates; DTNB, 5,5′-dithibis-(2-nitrobenzoic acid); GC-EI-MS, gas chromatography–electron ionization–mass spectrometry; GSH, glutathione; HPLC-UV, high-performance liquid chromatography with ultraviolet detection; ITCs, isothiocyanates; NAC, *N*-acetyl-L-cysteine

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2. Materials and methods

2.1. Chemicals

BITC was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). 1,2-Benzenedithiol and GSH were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). L-Cysteine was purchased from Ishizu Pharmaceutical (Osaka, Japan). *N*-Acetyl-L-cysteine (NAC) and 5,5′-dithibis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *S*-(*N*-Benzylthiocarbamoyl)-L-cysteine was obtained from Abcam (Cambridge, MA, USA). All other chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) or Nacalai Tesque Inc. (Kyoto, Japan) with analytical grade.

2.2. Extraction of BITC from papaya by distillation

The green papaya was purchased from a local market in Okinawa, Kanagawa and Hiroshima, Japan, in April 2016, June 2018, May 2019 and June 2019. The edible part of the green papaya (250 g) was placed in a commercial blender (IFM-C20G, Iwatani) and crashed for 1 min without adding water. After this process was repeated twice, the papaya pastes (final volume, 500 g) were heated using an oil bath at 100 °C, and the steam was gathered using a Liebig condenser with coolant water. The distilled water was collected and the yield was approximately 300 mL. The distilled water were analyzed by a gas chromatography–electron ionization–mass spectrometry (GC-EI-MS) system (Shimadzu GCMS-QP2010 plus) equipped with a 60 m × 0.25 mm Rtx-5MS column. The injector was used in the splitless mode, and the scan range was from m/z 50 to m/z 1000. Sample injection volume was 1 μL. The temperature of the column oven was held at 50 °C for 5 min before being raised to 300 °C at the rate of 10 °C/min, which was then held constant for 5 min.

2.3. Stability of BITC under aqueous conditions

The distilled water from the green papaya was stored at 4, −20 and −80 °C in 1.5 mL tubes for the indicated periods. To investigate the effect of the thiol compounds, such as L-cysteine, NAC and GSH, on the BITC stability, BITC in aqueous solution was prepared as described previously (Ohta, Takatani, & Kawakishi, 2000) with some modifications. Briefly, authentic BITC dissolved in DMSO (50 mM) was diluted in distilled water (the final BITC and DMSO concentrations of 50 μM and 0.1%, respectively). L-Cysteine, NAC or GSH (1 mM) was added to this water solution. These samples were stored at 4 °C for the indicated periods.

2.4. Cyclocondensation assay

The concentrations of BITC in the aqueous solutions were measured by a cyclocondensation assay as previously reported (Zhang, Cho, Posner, & Talalay, 1992) with some modifications. Briefly, the samples (150 μL) were incubated with 250 μL of 8 mM 1,2-benzenedithiol in ethanol and 100 μL of 100 mM potassium phosphate buffer (pH 8.5) for 2 h at 65 °C. After incubation using a water bath, the reaction solutions were centrifuged at 19,000 × *g* at 4 °C for 5 min using a high-speed refrigerated micro centrifuge (TOMY MX-305). The supernatants containing the reaction product, 1,3-benzodithiole-2-thione, were analyzed by reverse-phase high-performance liquid chromatography with ultraviolet detection (HPLC-UV) at 365 nm (Waters ACQUITY UPLC H-Class). The HPLC separation was done with isocratic elution (0.1% formic acid/acetonitrile = 20/80) using an ACQUITY UPLC BEH C18 (2.1 × 50 mm) column (Waters, Milford, MA, USA) at the flow rate of 0.4 mL/min with a column oven temperature of 40 °C. Sample injection volume was 2 μL. The limit of detection for 1,3-benzodithiole-2-thione was 100 nM.

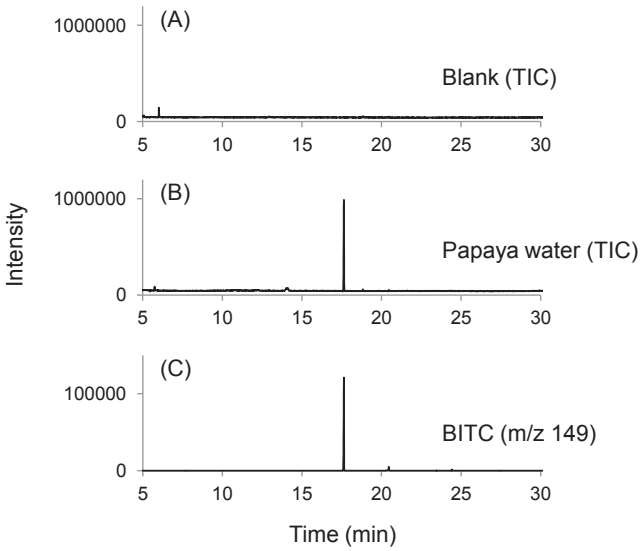

Fig. 1. Total ion chromatograms of blank (A) and the distilled papaya water (B), and selected ion monitoring of BITC (C).

2.5. Analysis of the conjugates of BITC and L-cysteine, NAC and GSH

The conjugation of BITC with L-cysteine, NAC or GSH in the distilled water was determined by reverse-phase HPLC-UV at 254 nm (Waters ACQUITY UPLC H-Class) as described previously (Conaway, Krzeminski, Amin, & Chung, 2001) with some modifications. Briefly, the HPLC separation was done by a gradient system using solvent A (0.1% formic acid) and solvent B (acetonitrile) and an ACQUITY UPLC BEH C18 (2.1 × 50 mm) column at the flow rate of 0.4 mL/min with a column oven temperature of 40 °C. Sample injection volume was 2 μL. The gradient program was 0 min (A 85%), 0.5 min (A 85%), 4.4 min (A 0%), 4.9 min (A 0%), 5.0 min (A 85%), and 6.0 min (A 85%). The concentrations of these conjugates were calculated as equivalent to the authentic standard of *S*-(*N*-benzylthiocarbamoyl)-L-cysteine.

2.6. Measurement of the free thiol groups of L-cysteine, NAC and GSH in the water containing BITC

The amounts of free thiol in L-cysteine, NAC and GSH in the water containing BITC were measured using DTNB as described previously (Nakamura, Kawai, Kitamoto, Osawa, & Kato, 2009) with some modifications. Briefly, DTNB (10 mM) was dissolved in 50 mM phosphate buffer (pH 7.4). The DTNB solution (10 μL) was mixed with 10 μL of L-cysteine, NAC or GSH in the water containing BITC in 480 μL of the 50 mM phosphate buffer (pH 7.4). After incubation for 15 min at room temperature in the dark, an aliquot (200 μL) was measured at 412 nm using a microplate reader (Benchmark Plus, Bio-Rad). The concentration of free thiol was calculated from the molar extinction coefficient (14,100 $M^{-1}L^{-1}$).

2.7. Statistical analyses

All values were presented as means ± S.D. Statistical analyses comparing the thiol compound supplementation groups with the untreated group (Day 1) were performed by Student's *t*-test, and comparison of the BITC purity between the green papaya samples was evaluated by chi-square test, using Microsoft Excel software (version 14.7.3).

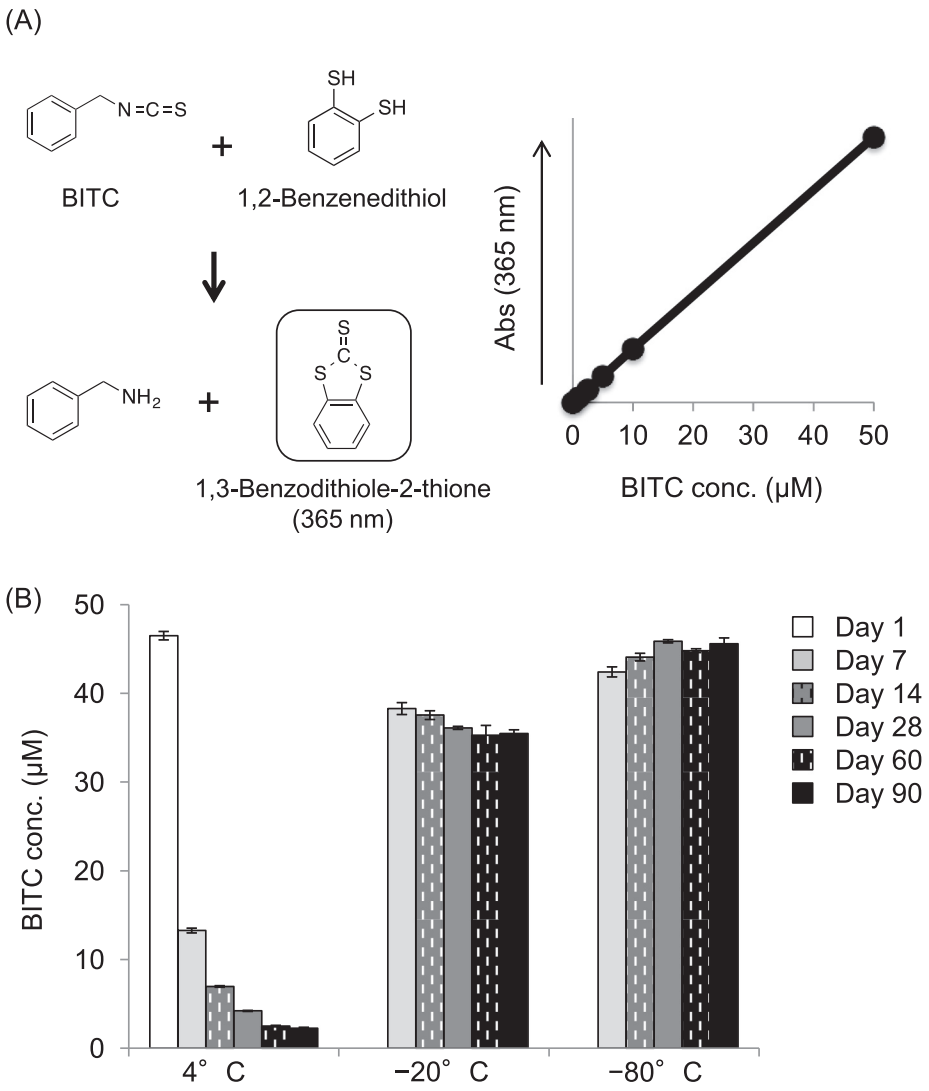

Fig. 2. Time-dependent changes in the BITC levels in the distilled water during the 4 °C-, −20 °C- or −80 °C-storage. The concentrations of BITC in the distilled water was measured by a cyclocondensation assay. The formation of the reaction product, 1,3-benzodithiole-2-thione, was determined by reverse-phase HPLC-UV at 365 nm. (A) Standard curve of 1,3-benzodithiole-2-thione and (B) time-dependent changes of the BITC levels in the distilled water. All values were expressed as means ± SD of four separate experiments.

3. Results

3.1. Extraction of BITC from mashed green papaya by distillation

The volatile compounds were extracted from the edible part of the green papaya using a Liebig condenser. The contents of BITC and other volatiles in the distilled water from the mashed green papaya were measured by GC–MS. As shown in Fig. 1, from the extract of the green papaya purchased in Okinawa, BITC was detected as a major peak with one minor peak, probably due to benzyl nitrile, a degradation product of BITC. The content percentage of BITC in this green papaya was estimated to be 85.6% based on the peak area, and the concentration of BITC in the distilled water was calculated to be 45 μM. BITC was also detected as a major peak with almost same purity (85.6 ± 3.0%) from all the green papaya fruits used in this study, in spite of the purchased regions and seasons. The statistical analysis by chi-square test revealed that there were no differences in the purity of BITC between the green papaya samples.

3.2. Storage time-dependent change in the BITC level in the distilled water

The time-dependent changes in the BITC amount in the distilled water from the papaya during storage were determined by HPLC-UV with the cyclocondensation reaction using 1,2-benzenedithiol. The standard curve of 1,3-benzodithiole-2-thione, a product of the cyclocondensation assay, is shown in Fig. 2A. The calculation data using this standard curve showed that BITC in the distilled water rapidly diminished over time, and it was hardly detected 90 days after storage at 4 °C (Fig. 2B). At 90 days, the BITC level in the distilled water was estimated at only 2.24 ± 0.12 μM.

BITC is considered to be unstable in an aqueous solution because of its decomposition by the reaction with a hydroxyl ion (Kawakishi & Namiki, 1969; Pecháček et al., 1997). Next, the stabilities of BITC in the water at −20 and −80 °C were investigated. The degradation of BITC was effectively prevented by each low temperature storage (Fig. 2B).

3.3. Effect of L-cysteine, NAC or GSH on the BITC stability in the distilled water

To improve the BITC stability, excess amounts of the thiol compounds, L-cysteine, NAC or GSH, were added to BITC in the distilled water. As shown in Fig. 3A, the addition of L-cysteine effectively prevented BITC from decomposition during 4 °C storage, and the amounts of BITC were maintained during storage for 14 days. In contrast, the

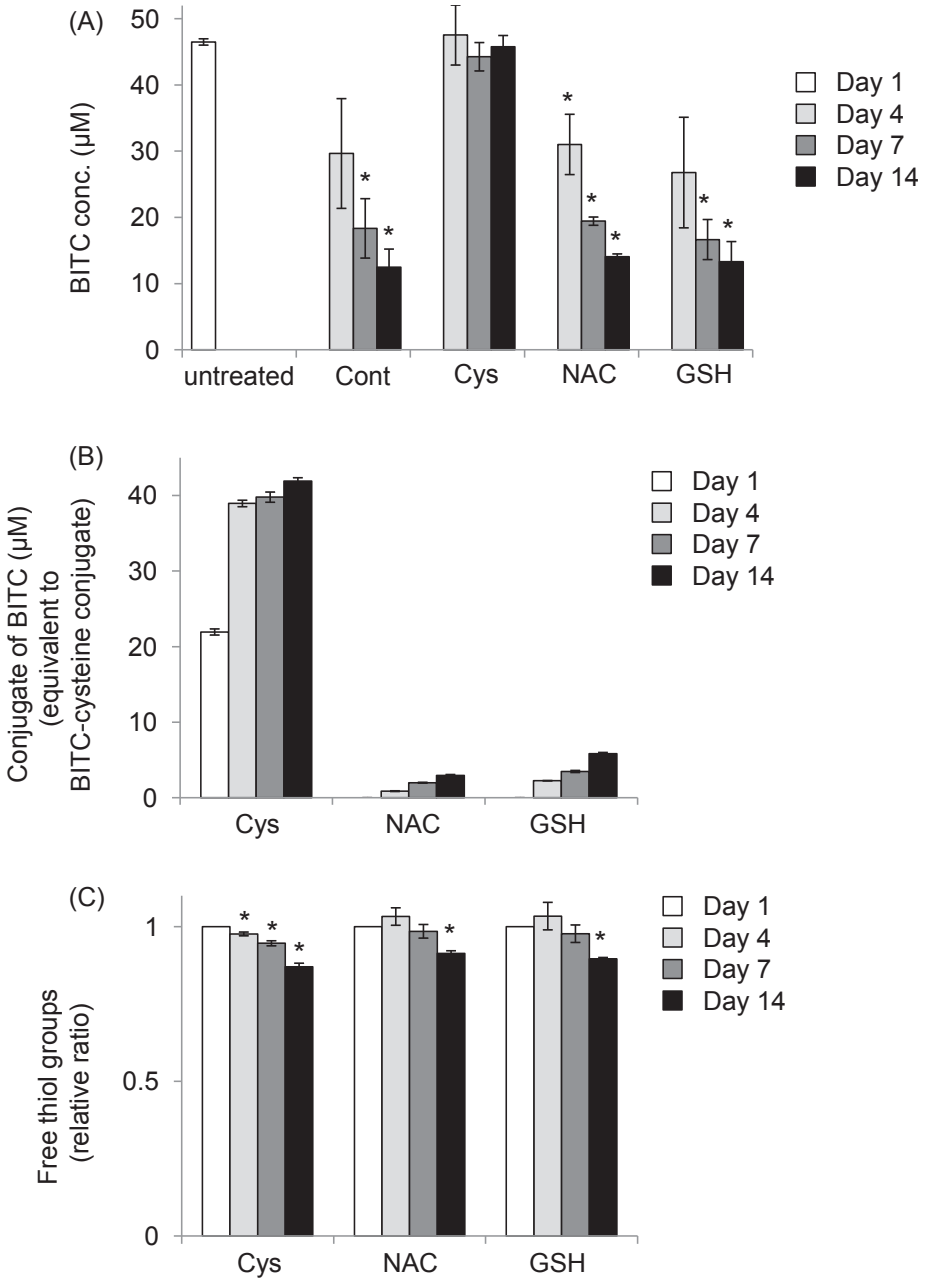

Fig. 3. Modulating effects of thiol compounds on the BITC stability in the distilled water. (A) Effects of L-cysteine, NAC and GSH on the BITC stability. The concentrations of BITC in the distilled water were measured by a cyclocondensation assay with the HPLC-UV analysis. (B) The conjugate formation of BITC with L-cysteine, NAC and GSH in the distilled water. The formed conjugates were analyzed by reverse-phase HPLC-UV at 254 nm. Their concentrations were estimated using the standard curve of the authentic *S*-(*N*-benzylthiocarbamoyl)-L-cysteine, which is equivalent to the other conjugates. (C) Stability of the free thiol groups in the thiol compounds during storage. The amounts of free thiol groups of L-cysteine, NAC and GSH in water were measured by a DTNB assay. All values were expressed as means ± SD of three separate experiments. An asterisk indicates significant difference between the thiol compound supplementation groups and the untreated group ($P < 0.05$).

BITC amount gradually declined in the presence of NAC or GSH comparable to the control group. The 5-fold higher concentration of NAC or GSH did not inhibit the BITC degradation (data not shown). HPLC analysis demonstrated that, in the presence of L-cysteine, BITC existed as the conjugated form with L-cysteine in the distilled water (Fig. 3B). On the other hand, NAC and GSH were hardly conjugated with BITC in the water, even though the free thiol groups of NAC and GSH as well as L-cysteine were not degraded but still reactive in the distilled water (Fig. 3C).

4. Discussion

In this study, BITC was successfully extracted as a major component with more than 80% purity from the mashed green papaya without using an organic solvent. This study is, to the best of our knowledge, the first report to extract BITC by distillation from plant food materials. Since several components have been detected in the papaya (Flath & Forrey, 1977), the difference of physico-chemical properties between BITC and other constituents, such as polarity and volatility, might allow the highly selective extraction for BITC. It was also confirmed that BITC in the distilled water has a potency to induce the drug metabolizing enzyme genes, heme oxygenase 1 and NAD(P)H quinone oxidoreductase 1, using the cell culture medium (minimum essential medium alpha powder (Gibco, NY, USA) with 10% fetal bovine serum) made of the distilled water from green papaya (papaya water medium) (Supplemental Fig. 1), similar to that of the authentic BITC (Liu et al., 2017). The stabilities of BITC in the distilled water and in the medium were determined by not only its biological activity but also the quantification of BITC (Fig. 2B and Supplemental Fig. 2). Expectedly, BITC in the water solution is very unstable and gradually disappeared during storage. Although the medium contains some nutritional components, such as amino acids and proteins, the BITC stability was not changed in the water and in the medium for 28 days (Supplemental Fig. 2). Moreover, BITC was more stable at −80 °C than that at −20 °C (Fig. 2B), suggesting that the temperature is one of the most important determinants for the BITC stability in the water.

Interestingly, the decomposition of BITC in the distilled water was prevented by the addition of L-cysteine, but not NAC and GSH (Fig. 3A). It has been reported that allyl ITC (AITC) was decomposed by thermal treatment, which was influenced by the pH condition (Chen & Ho, 1998). Ohta *et al.* reported that the decomposition of AITC in an aqueous solution was retarded by inclusion complexation within the cavities of α-cyclodextrin (CD) and β-CD (Ohta et al., 2000). The suppression mechanism of the decomposition of BITC and phenyl ITC by CD might be almost the same as the AITC-α-CD complex (Ohta, Matsui, Osawa, & Kawakishi, 2004). There is, however, no report on the effect of thiol compounds on the BITC stability in aqueous solutions, even though thiol compounds used in this study are well known to primarily react with BITC *in vivo* and *in vitro* (Hong, Freeman, & Liebler, 2005; Zhang, 2000). As shown in Fig. 3B, the formation of the BITC-cysteine conjugate in distilled water was observed for 14 days, whereas the BITC-GSH and BITC-NAC were detected only at a low level at all times. The much lower levels of the NAC or GSH conjugates might not be due to lack of thiol groups, because decreases of free thiol groups in L-cysteine, NAC and GSH in the distilled water were very slight during the incubation (Fig. 3C). Although the reason why BITC hardly reacts with NAC and GSH in an aqueous solution remains to be determined, L-cysteine prevents BITC from its decomposition in an aqueous solution, possibly through the conjugate formation.

The ITC exposure to the cells leads to the rapid and high intracellular accumulation mainly through simple diffusion. The BITC-GSH conjugate is produced as the primary metabolite after BITC was absorbed *in vivo* (Nakamura et al., 2018). BITC-GSH is stepwise metabolized to BITC-cysteinylglycine, BITC-cysteine, and BITC-NAC *via* the mercapturic acid pathway. Although BITC is present in our body as conjugated forms, the conjugates have similar biological activities with the intact ITCs (Conaway et al., 2005). Taken together with its lower toxicity than intact BITC, the combination with L-cysteine is one of the promising strategies to enhance the BITC stability in an aqueous solution.

In conclusion, this study conducted a new extraction method of BITC from the mashed green papaya using distillation apparatus. The volatile BITC can be extracted from food material without using organic solvents. In addition, the effects of different temperatures and thiol supplementation on the stability of BITC in aqueous solution were investigated. Finally, the decomposition of BITC could be inhibited by the frozen storage or the addition of L-cysteine. The present results support the idea that the distillation of mashed green papaya might be a useful method for the preparation of the safer grade of BITC-containing extract.

Acknowledgements

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125118.

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Further reading

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