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Suppression of Volatile Sulfur Compounds from Broccoli by Freeze-Thawing and Their Mechanism with Filtration and Centrifugal Fractionation

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Abstract

The formation of methanethiol and dimethyl disulfide in the broccoli tissue homogenates following filtration and centrifugal fractionation, and their suppression by freeze-thawing was investigated. Methanethiol was formed only in the headspace of the residue but not in the filtrate or supernatant fractions like dimethyl disulfide. Freeze-thawing, however, suppressed their formation in all homogenate fractions. The immersion of the fresh residue in a liquid medium decreased the concentration of methanethiol which indicates that this compound is highly likely soluble in water and produces only in the headspace of the environment wherein the condition is free of liquid medium. Hence, methanethiol formation was not possible except for dimethyl disulfide after addition of the co-factor, pyridoxal 5-phosphate, in the frozen residue. Both compounds were also inhibited by aminooxyacetic acid, a potential inhibitor of pyridoxal phosphate-dependent enzymes, which indicates that MT and DMDS are produced upon the action of cysteine sulfoxide lyase. This enzyme, which yielded strong activity in the residue upon extraction with buffer containing Triton X-100, is highly likely to be a bound enzyme. Suppression of methanethiol and dimethyl disulfide during freeze-thawing is likely attributed to the retardation of the enzyme action due in part to the loss of the co-factor, pyridoxal 5-phosphate and its substrate, S-methyl-L-cysteine sulfoxide and owing to the solubility of methanethiol and its affinity for gaseous condition.

Key Words: broccoli, cysteine sulfoxide lyase, dimethyl disulfide, freeze-thawing, methanethiol, residue fraction.

Introduction

Broccoli (*Brassica oleracea* L. var. *italica* Plenck) and other *Brassica* vegetables produce many volatile sulfur compounds upon tissue disruption or maceration (Chin and Lindsay, 1993; Marks et al., 1992). These sulfur compounds are important in imparting the characteristic flavor and odor of these vegetables (Buttery et al., 1976). Recently, these volatiles are also of interest to investigators due to their anticarcinogenic properties (Marks et al. 1992; Miller and Stoewstand, 1983; Wargovich and Goldberg, 1985). The main volatile sulfur compounds already identified in broccoli are methanethiol (MT) and dimethyl disulfide (DMDS) (Dan et al., 1997; Di Pentima et al., 1995; Forney and Jordan, 1999; Forney et al., 1991; Hansen et al., 1992).

The emission of MT in higher plants has been reported to occur in response to methionine and S-methyl-L-cysteine sulfoxide. (Schmidt et al., 1985; Di Pentima et al., 1995). Chin and Lindsay (1994) suggested that enzymatic breakdown of S-methyl-L-cysteine sulfoxide upon the

action of cysteine sulfoxide lyase (C-S lyase) may generate MT. Recently, Dan et al. (1997) and Obenland and Aung (1996) support the view that C-S lyase are involved in the biosynthesis of anaerobically induced MT and DMDS from broccoli florets.

In our previous work (Tulio et al., 2002), we investigated the formation of the main volatile sulfur compounds, MT and DMDS, in broccoli florets following manual crushing, homogenizing and freeze-thawing. Our findings showed that these objectionable odors were formed upon the gradual disruption through manual crushing of the fresh tissues, whereas rapid disruption through homogenization of the tissues produced only DMDS. However, freeze-thawing of the broccoli tissues almost completely inhibited the formation of these compounds.

The present study was undertaken to elucidate the factors associated with the suppression of MT during rapid homogenization. Specifically, the location for the formation of this sulfur volatile together with DMDS in various homogenate fractions such as residue and filtrate after squeezing with gauze, and supernatant and precipitate after centrifugal fractionation was determined. Freeze-thawing of the broccoli homogenate fractions was also conducted to investigate its inhibitory effect on the formation of volatile sulfur compounds.

Materials and Methods

Plant Material

Fresh broccoli (*Brassica oleracea* L. var. *italica* Plenck) heads were procured from a local supermarket in Osaka, Japan. Broccoli buds measuring 2-3 mm long were excised using a stainless knife from the broccoli heads and used as the main material in this study.

Sample Preparation

Fresh Homogenate Fractions. Fresh broccoli buds were directly homogenized in 0.1 M sodium phosphate buffer solution (pH 8.0) by using a Vortex homogenizer (IKA Laboratory Technology Ltd., Japan). The ratio (w/v) of broccoli buds to buffer solution was 1:2. The homogenate was filtered through four layers of gauze. An adequate amount of the residue (6-7 g) and filtrate (20-22 ml) were collected and placed separately in a 100 ml-Erlenmeyer flask. The remaining residue was set aside, whereas those of the filtrate was further centrifuged at 10,000 × g for 20 min at 4℃. After centrifugation, the supernatant was collected and approximately 20-22 ml of which was transferred in a 100 ml-Erlenmeyer flask. The remaining precipitate was also placed in another flask. Three replicates were prepared for each homogenate fraction.

Frozen Homogenate Fractions. Fresh broccoli buds (20 g) in 100 ml-Erlenmeyer flask were frozen using liquid nitrogen and stored at -80℃ for 24 h. The flask was placed in tap water for about 2 min to slightly thaw the frozen tissues contained inside the flask. Complete thawing was undertaken during homogenization of the frozen tissues in sodium phosphate buffer. Homogenization, filtration, and centrifugation of the frozen broccoli tissues together with sample collections were conducted following the procedure in the preceding experiment.

After that, each flask was sealed with silicon stoppers and incubated for 2 h at 30℃ in a water bath prior to headspace gas analysis by using GC-FPD.

Buffer and Enzyme Co-factor Treatments

Fresh residue in 100 ml-Erlenmeyer flask was prepared similar to the procedure in the earlier experiment. Twice the volume (w/v) of 0.1 M phosphate buffer solution was added in the test treatment, whereas the control sample was not treated with buffer solution.

Frozen-thawed residues were prepared following the described method for frozen homogenate fraction samples. Test flask containing 7 g of frozen-thawed residue was treated with 14 ml of 10 mM pyridoxal 5-phosphate (PALP) while same volume of distilled water was added in the control flask.

Silicon stoppers were used to seal the flasks after the treatments. The flasks were incubated for 2 h at 30℃ in a water bath and headspace gas was analyzed by using GC-FPD.

Inhibition Assay

Fresh broccoli buds (70 g) were homogenized in 140 ml of 0.1 M sodium phosphate buffer (pH 8.0) using a Vortex homogenizer at the highest speed for 1-2 min. The homogenate was filtered through 4 layers of gauze and the residue fraction was collected and divided into two parts.

Inhibition assay was performed with aminooxyacetic acid (AOA), a potential inhibitor of pyridoxal phosphate-dependent enzymes, on the residue following the slightly modified procedure of Derbali et al. (1998). The first part of the residue fraction (test treatment), approximately 25 g, was soaked in 100 mL of 2 mM AOA for 1-2 min while subjecting to vacuum. The AOA solution was filtered through a Buchner funnel and the residue fraction was transferred in a 100 ml-Erlenmeyer flask. The flask was sealed with silicon stopper and incubated for 2 h at 30℃ in a water bath. The remaining residue fraction (control) was soaked in 100 ml distilled water instead of AOA and incubated in the same manner prior to headspace analysis using GC-FPD.

GC-FPD Headspace Gas Analysis

MT and DMDS, which accumulated in the headspace of the flasks, were analyzed by using a gas chromatograph with a flame photometric detector (GC-FPD). Two milliliters of headspace samples were withdrawn from the flasks after holding for 2 h at 30℃ with a gas-tight syringe and injected into a gas chromatograph (Hitachi model 163, Hitachi Co. Ltd., Tokyo, Japan). The volatile sulfur compounds were separated on two glass columns connected in series. The conditions and descriptions of the columns were described in previous report (Tulio et al., 2002). Peaks were identified by comparing the retention times of the samples with those of standard MT and DMDS.

Crude Enzyme Extraction

Fresh broccoli tissues (20 g) was homogenized in 40 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 3% (w/v) polyvinylpyrrolidone using a Vortex homogenizer at the highest speed for 2-3 min. The homogenate was filtered through 4 layers of gauze and the residue was collected. About 200 μg of the residue was used directly used as crude sample for enzyme assay. The supernatant was obtained by centrifuging the filtrate at 10,000×g for 20 min at 4℃ and desalted on Sephadex G-25 to remove any pyruvates in the extract which could interfere in the enzyme assay. The desalted supernatant was collected and 200 μl was used as crude extract.

The remaining fresh residue (7 g) was further homogenized in 14 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing Triton X-100 in various concentrations (0, 0.1, 1.0, and 5.0%) (v/v) using a Vortex homogenizer at the highest speed for 2-3 min. The homogenate was filtered through 4 layers of gauze again and centrifuged at 10,000×g for 20 min at 4℃. The supernatant was collected and desalted in Sephadex G-25. Then, 200 μl of the supernatant was used as the crude enzyme solution.

Residue from the fresh broccoli tissues was prepared similar to the procedure mentioned above. Acetone-dried powder from fresh residue was conducted subsequently following the method of Nelson (1955) with slight modification. About 20 g of fresh residue was homogenized in 100 ml of chilled acetone using a Vortex homogenizer at the highest speed for 2-3 min. The resulting slurry was filtered through a Buchner funnel and washed several times with chilled acetone. The residue spread out on filter paper was dried with diethyl ether and placed in a desiccator for approximately 24 h with continuous aeration. The acetone powder was stored at -20℃ prior to enzyme assay.

Crude enzymes were also extracted from frozen broccoli tissues following the freeze-thawing process described earlier. All crude enzyme extractions and assays were done in triplicates.

Enzyme Assay

C-S lyase activity was assayed following the procedure described by Hamamoto and Mazelis (1986) with slight modification. The standard reaction mixture contained 0.1 M sodium phosphate buffer (pH 8.5), 40 mM S-methyl-L-cysteine sulfoxide (SMCSO), 0.25 mM pyridoxal 5-phosphate (PALP), and crude enzyme solution in a total volume of 1.0 ml. The reaction mixture was incubated at 30℃ in a water bath for 10 min. The reaction was terminated by the addition of 2 ml of 10% trichloroacetic acid and centrifuged at 3,000×g for 10 min. An aliquot of the supernatant was assayed for pyruvate colorimetrically by the total keto acid method of Friedemann and Haugen (1943).

The activity of the C-S lyase from acetone-dried powder of the residue fraction was assayed following the procedure mentioned above. Before analysis, about 400 mg of acetone-dried powder was suspended in 20 ml of 0.1 M

sodium phosphate buffer for 1 h in ice water bath. The solution was then centrifuged at 10,000×g for 20 min at 4℃ and filtered with a single gauze. About 200 µl of the crude extract was used for enzyme assay.

Protein Measurement

Protein content of the samples was estimated according to the dye-binding procedure described by Bradford (1976) using bovine serum albumin as the standard.

Extraction of *S*-Methyl-L-Cysteine Sulfoxides

The extraction of S-methyl-L-cysteine sulfoxides (SMCSO) from fresh broccoli tissues was carried out according to the method previously described by Tulio et al. (2002) with slight modification. Thirty grams of broccoli buds were homogenized in 60 ml of 0.1 M sodium phosphate buffer (pH 8.0) using Vortex homogenizer at the highest speed for 1-2 min. The residue was collected after the homogenate was filtered through 4 layers of gauze. The supernatant was also collected after the filtrate was centrifuged at 10,000×g for 20 min at 4℃. Residue (7 g) and supernatant (22 ml) fractions were placed individually in Erlenmeyer flask. Two batches of both residue and supernatant fractions were prepared and incubated at 30℃ for 0 and 2 h, respectively. In addition, residue and supernatant from frozen-thawed tissues were prepared following the procedure described elsewhere.

The residue was extracted with 24 ml of 99% ethyl alcohol by using a reflux tubes for 15 min at boiling temperature, whereas 98 mL of 99% ethyl alcohol was used to extract the supernatant. The extract was filtered and washed twice with 80% chilled ethyl alcohol. The filtrate was made up to 100 ml volume with 80% chilled ethyl alcohol. Twenty milliliters of the alcohol extract was evaporated at 40℃ using a rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan) and dissolved in 10 mL of 0.1 N HCl. The aliquot was passed through a 0.45-µm cellulose nitrate membrane filter (Advantec, Toyo Roshi Kaisha, Ltd., Japan).

About 100 µL of this aliquot was injected into the amino acid analyzer (Model K-101, Kyowa Seimitsu Co., Ltd., Japan) equipped with a visible monitor (Model KLC-450), sequence controller and thermal programmer (Model KTC-104). The analyzer was also equipped with a 4.6×150 mm (i.d.) column packed with 62210 F (Na^+) (Kyowa Seimitsu Co., Ltd., Japan) and three elution buffers: buffer 1, 0.2 N sodium citrate (pH 3.18); buffer 2, 0.2 N sodium citrate (pH 4.25); and buffer 3, 2.0 N sodium citrate (pH 4.45). The oven temperature was 55℃ and the flow rate was 0.5 ml/min. Peaks were identified and quantified by comparing the retention times with those of standard SMCSO.

Results and Discussion

MT and DMDS Production and Its Suppression by Freeze-thawing

The location for the formation of the main volatile sulfur compounds, MT and DMDS, in fresh and frozen-thawed broccoli tissues was investigated following filtration and centrifugation. These homogenate fractions are residue, filtrate, supernatant, and precipitate (Fig. 1). Results showed that after incubation for 2 h at 30℃, MT was formed in marked quantities only in the residue among the different homogenate fractions (Fig. 1A). In contrast, production of DMDS was not only detected in the residue but also in other homogenate fractions such as filtrate, supernatant, and precipitate. More than 30 nmol/g fresh weight of MT were detected in the residue, whereas DMDS formed in the same residue was approximately 20 nmol/g fresh weight only. In addition, the amount of DMDS formed in the filtrate and supernatant fractions was 3.12 and 1.65 nmol/g fresh weight, respectively, whereas the DMDS in the precipitate fraction was 0.08 nmol/g fresh weight only and almost negligible.

However, freeze-thawing of the residue fraction from fresh broccoli tissues completely inhibited the production of MT and DMDS compounds (Fig. 1B). Formation of DMDS in the filtrate, supernatant and precipitate fractions was also completely inhibited after freeze-thawing the tissues of broccoli bud samples. These findings indicate that freeze-thawing has a dire consequence on the formation of both sulfur compounds, MT and DMDS, in the residue and the production of DMDS in other homogenate fractions.

Buffer and Enzyme Co-factor Treatment

Immersion of the fresh residue to the sodium phosphate buffer solution had a severe suppressing effect on the formation of MT. However, the formation of DMDS compound was affected very slightly (Fig. 2). Production of MT in the residue fraction of broccoli buds with buffer treatment was reduced by 3-fold after 2 h of incubation at 30℃. This indicates that MT is likely soluble in water (23.3 g/l at 20℃) (Merck Index, 1996) and this compound seems to produce only in the headspace of the environment wherein the condition is not fully-covered by the liquid medium.

In Figure 3, the addition of PALP in the residue fraction of frozen-thawed broccoli tissues enhanced the formation of DMDS but not MT. About 12.33 nmol/g fresh weight of DMDS was recovered. In contrast, only 3.23 nmol/g fresh weight was formed when only distilled water was added instead of PALP. The formation of MT in the headspace of the frozen residue tissue was not detected after PALP addition due probably to the aversion of this compound to liquid medium similar to the results demonstrated earlier in Figure 2. Furthermore, the inhibition of MT in the frozen-thawed residue must be likely due to its extreme high volatility (with a boiling point of 6℃ at atmospheric pressure)

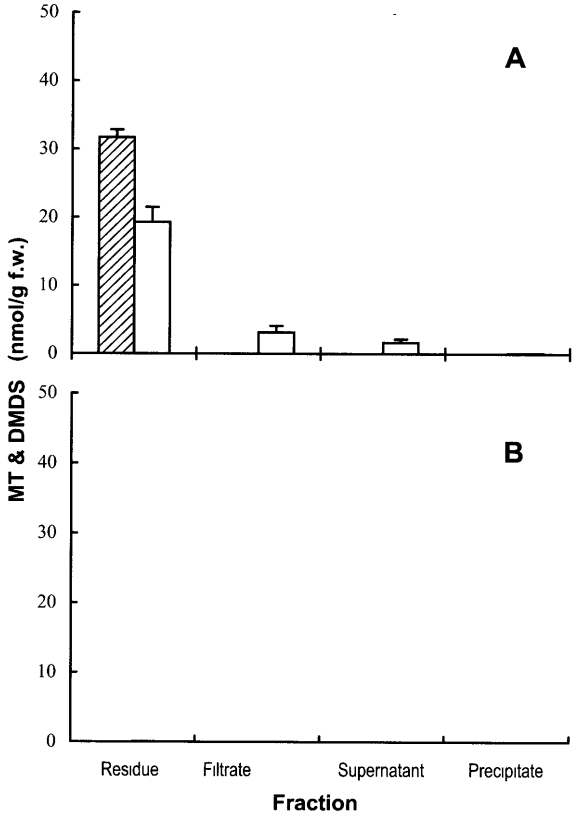

Fig. 1. Production of MT (slashed bar) and DMDS (white bar) in homogenate fractions of (A) fresh broccoli buds and their inhibition in (B) frozen-thawed broccoli tissues after 2 h of incubation at 30℃.

Bars are mean ± SD (n = 3). Unshown bar means not detected. f.w., fresh weight.

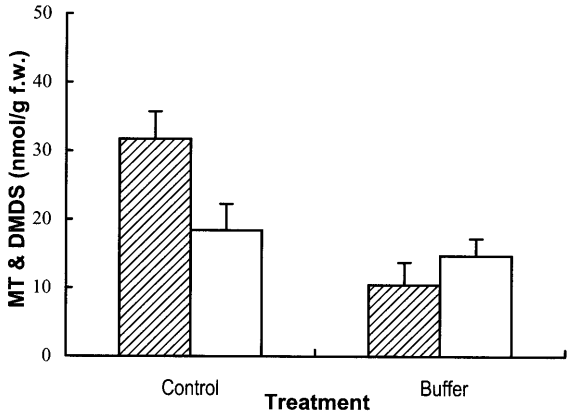

Fig. 2. Production of MT (slashed bars) and DMDS (white bars) in the residue fraction of fresh broccoli tissues with or without (control) buffer treatment after 2 h of incubation at 30℃.

Bars are mean ± SD (n = 3). f.w., fresh weight.

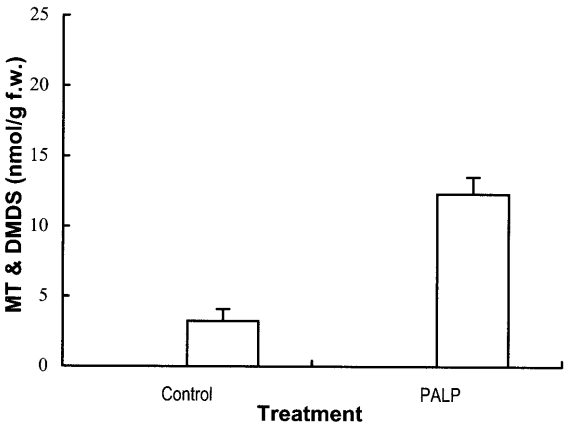

Fig. 3. Production of MT (slashed bar) and DMDS (white bars) in residue fraction of frozen-thawed broccoli tissues with distilled water (control) or pyridoxal 5-phosphate (PALP) treatment after 2 h of incubation at 30℃.

Bars are mean ± SD (n = 3). Unshown bar means not detected. f.w., fresh weight.

(Buttery et al., 1970).

Hence, freeze-thawing of the residue must have hindered the production not only of the DMDS compound but also MT due to the loss of the co-factor, pyridoxal 5-phosphate. This result indicates that C-S lyase requires exogenous PALP for the stimulation of its activity in the residue fractions of the frozen broccoli buds. The result also suggests that this enzyme is not the only limiting factor in the production of sulfur volatiles in the broccoli residue fraction.

Nature of C-S Lyase in Frozen-Thawed Residue

Aminooxyacetic acid (AOA), which is a potential inhibitor of pyridoxal phosphate-dependent enzymes, was used to investigate its effects on the production of MT and DMDS in the residue fraction of the fresh broccoli tissues. These sulfur volatile compounds were completely inhibited upon the AOA treatment in the residue fraction. This indicates that the objectionable odor is produced upon the action of C-S lyase on the substrate, S-methyl-L-cysteine sulfoxide (SMCSO) (Fig. 4). Derbali et al. (1988) also showed evidence in support of the enzymatic origin of the sulfur volatile compounds by leaf tissues in response to anaerobic treatment using AOA.

The activity of C-S lyase in the residue fraction of the frozen-thawed broccoli tissues had no marked difference with those from the fresh samples (Table 1). Likewise, the activity of this enzyme in the supernatant fraction from frozen-thawed broccoli tissues was almost similar to the fresh sample. However, more activity was detected in the supernatant than in the residue fractions, whether the tissue samples were frozen or not. The activity from the supernatant was more than 2-fold than those of the residue sample.

Further investigations showed that C-S lyase yielded strong activity from the residue upon extraction with buffer containing Triton X-100 (Table 2). The activity of this enzyme from the residue rose 2.4-fold in buffer with 0.1% Triton X-100, whereas 1.0 and 5.0% Triton X-100 in the extraction buffer had shown a 2.7- and 3.8-fold increase, respectively. The low activity in the residue was likely due to the bound part of the enzyme which was not readily released during extraction using regular buffer medium and remained in the membrane during extraction process. The enzyme in the supernatant was likely a free type, hence, it was easily detected during enzyme assay. Results also showed that remaining C-S lyase in the residue fraction was all released during enzyme extraction upon

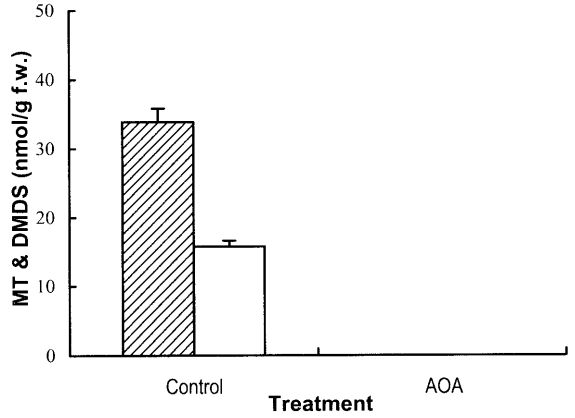

Fig. 4. Production of MT (slashed bar) and DMDS (white bar) in the residue fraction of fresh broccoli tissues with distilled water (control) or aminooxyacetic acid (AOA) treatment after 2 h of incubation at 30℃.

Bars are mean ± SD (n = 3) Unshown bar means not detected. f.w., fresh weight

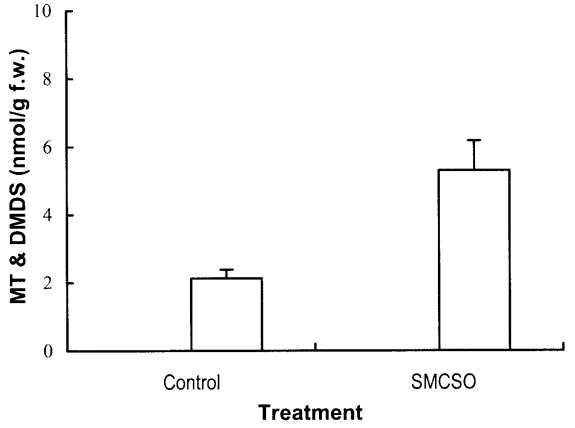

Fig. 5. Production of MT (slashed bar) and DMDS (white bars) in the residue fraction of frozen-thawed broccoli tissues with distilled water (control) or S-methyl-L-cysteine sulfoxide (SMCSO) treatment after 2 h of incubation at 30 ℃.

Bars are mean ± SD (n = 3). Unshown bar means not detected. f w , fresh weight.

Table 1. C-S lyase activity in the residue and supernatant fractions of fresh and frozen-thawed broccoli tissues

Homogenate fraction	Tissue treatment	C-S lyase activity [a] (μg/min/mg protein)
Residue	Fresh	0.62±0.09
	Frozen-thawed	0.51±0.11
Supernatant	Fresh	1.40±0.05
	Frozen-thawed	1.25±0.07

[a] Values are mean ± SD (n = 3).

Table 2. Effect of extractions on the C-S lyase activity in the residue fraction of fresh broccoli tissues

Extraction method	Triton X-100 treatment (%)	C-S lyase activity [a] (μg/min/mg protein)
Buffer	0.0	0.62±0.09
	0.1	1.46±0.29
	1.0	1.70±0.08
	5.0	2.38±0.07
Acetone	-	0.80±0.21

[a] Values are mean ± SD (n = 3).

Table 3. SMCSO contents of the residue and supernatant fractions of fresh and frozen-thawed broccoli tissues

Homogenate fraction	Tissue treatment	SMCSO (mg/100 g f.w.)[a]	
		Thawing time at 30℃ 0 h	2 h
Residue	Fresh	124.1±10.7	73.1±8.0
	Frozen-thawed	54.0± 8.4	18.5±6.1
Supernatant	Fresh	172.9±12.2	77.0±4.0
	Frozen-thawed	265.8±10.1	85.0±3.6

[a] Values are mean ± SD (n = 3). SMCSO, *S*-methyl-L-cysteine sulfoxide. f.w., fresh weight.

using Triton X-100. This indicates that a bound type of C-S lyase enzyme highly likely exists in the cell membranes of the residue tissue.

The use of chilled acetone as a precipitation medium, however, had no marked difference on the activity of C-S lyase in the residue (Table 2). The chilled acetone medium yielded only slightly more enzyme activity than those from regular buffer medium. Acetone may not be suitable for the extraction of C-S lyase and possibly this enzyme was denatured in the acetone-dried powder during extraction process.

Precursor, SMCSO, in the Frozen-thawed Residue

Table 3 shows the results of the SMCSO analysis conducted in the residue and supernatant fractions. As in the case of the enzyme activity, the SMCSO content in the supernatant was higher than those in the residue. In addition, the SMCSO content in the residue of the frozen-thawed broccoli tissues was only less than half than those residue from the fresh tissues. After 2 h of thawing at 30 ℃, the SMCSO content of the residue from frozen-thawed tissues decreased by almost 3-fold (Table 3). Inhibition of the volatile sulfur compounds in frozen-thawed residue may also be attributed to the low SMCSO contents which have a negative repercussion on the action of C-S lyase.

Results in Figure 5 showed that the addition of the precursor, SMCSO, in the frozen-thawed residue tissues led to the formation of DMDS but not MT after 2 h at 30℃. DMDS was detected in the frozen-thawed residue with SMCSO by 2.5 times compared with the control wherein the SMCSO was replaced with distilled water. This result confirms that freeze-thawing has a direct negative impact on the precursor which resulted in the retardation of the enzyme action prohibiting the production of the volatile sulfur compounds. The absence of the MT can be attributed to the condition of the frozen-thawed tissues similar to those previously discussed earlier.

In our previous work, rapid disruption of the fresh tissues by homogenization of broccoli florets produced significant quantities of DMDS but only trace amounts of MT. However, upon filtration and centrifugal fractionation of the homogenate, we found out that MT was considerably produced in the residue but not in the filtrate or supernatant fractions. We conclude that MT is not capable on surviving in the environment filled or covered with a liquid medium as shown by the suppression of MT upon the treatment of buffer solution in the residue of the fresh broccoli tissues. Therefore, formation of

MT compound can only occur in gaseous condition which is similar to the condition in crushed tissues and anaerobically-induced tissues (Tulio et al., 2002). Moreover, freeze-thawing of the residue caused the loss of pyridoxal phosphate and SMCSO which severely retarded the action of C-S lyase hindering the formation of MT and DMDS in the residue fraction of broccoli buds.

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