Optimisation of the Method for the Quantitative Determination of Sulforaphane in Broccoli

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Abstract

Consumption of vegetables, especially crucifers, reduces the risk of developing cancer. Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], a compound with the ability to inhibit carcinogenesis, is one of the degradation products of glucosinolates in cruciferous vegetables. Among available extraction methods, autolysis at room temperature is the most effective for Sulforaphane extraction (relatively higher purity and better yield). The research work undertaken at Federal Research Centre for Nutrition, Institute of Biology and Chemistry, Karlsruhe, Germany was isolation of Sulforaphane based on cruciferous vegetables like Broccoli (Brassica oleracea L. Cv. italica) employing autolysis – the yield being higher. The extracted Sulforaphane compound’s purity and yield were accordingly examined with gas chromatography.

Keywords: Optimisation: sulforaphane; broccoli
Introduction

Epidemiological studies indicate that a diet rich in cruciferous vegetables, such as broccoli, cabbage, Brussels sprouts, cauliflowers, kale, kai Choi and watercress, can reduce the risk from a number of cancers (Fenwick, 1983; Graham, 1983; Wattenberg, 1993; Talalay and Zhang, 1996; Kohlmeier and Su, 1997). These vegetables are rich in sulfur-containing glucosides called glucosinolates which exhibit minimal anticancer activity. These compounds, upon hydrolysis by the *endogenous enzyme myrosinase* (thioglucoside glucohydrolase EC 3.2.3.1), yield a variety of bioactive products, including isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones, sulfate and D-glucose, depending on the chemical structure of parent glucosinolates and the conditions, such as temperature and pH, during enzymic cleavage (Cole, 1976; Fenwick et al. 1989; Bones and Rossiter, 1996).

Among these, sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], which has been identified in broccoli (a member of the Brassicaceae) as a product of enzymatic- or acid hydrolysis of the corresponding ω-(methylsulfinyl)-alkyl-glucosinolate (glucoraphanin), has aroused a great deal of interest (Zhang et al. 1992b; Kore et al. 1993). Sulforaphane is a potent inducer of phase (II) enzymes, including quinone reductase and glutathione S-transferases, which protect against carcinogens and other toxic electrophiles (Zhang et al. 1992 & 1994).

The undesirable part of the hydrolysed products is its pungent odour, bitterness and toxic or goitrogenic activity and thus clearly indicate that control of myrosinase activity in cruciferous vegetables is highly desirable (Ludikhuyze, 1999). Thermal treatments were pre-eminently used to control the enzyme activity but resulted in considerable losses of sensorial (taste, flavor, color) or nutritional quality attributes (Lund, 1977). Consequently new methods for controlling enzymic activity are being investigated, including hydrostatic pressure (Oga-wa et al., 1990:...
The emphasis of the present research work was to develop the method of sulforaphane determination in cruciferous vegetables, including the extraction of sulforaphane and quali-quantitative analysis. Gas Chromatography was used to confirm the presence of sulforaphane in sample extracts and to validate the new methods. The effect of temperature- and pressure treatment, and microwave cooking on the extent of sulforaphane were also investigated to optimise the conditions of enzymic cleavage in terms of maximum sulforaphane content.

The present enzymatic hydrolysis technique of sulforaphane extraction from broccoli and its follow up quali-quantitative tests would also enhance the Myanma indigenous medicinal compounds extraction practices from medicinal plants – in terms of yield, purity and nutritive value at competitive costs.

Materials And Methods

Materials

Fresh broccoli (Brassica oleracea L. Cv. italica) of commercial maturity was purchased from local markets in Germany. Thioglucosidase (S-5376) was purchased from Sigma Chemical Co. (St. Louis, MO). Sulforaphane was obtained from ICN Biomedical Inc. All other chemicals were of analytical grade.

Instruments

A SPB-5 (Supelco) model gas chromatograph, equipped with the column, dimensions being 15 m in height, 0.25 mm inside diameter and a film thickness of 0.25 micron. A water bath shaker was used in all incubations. Ultra Turrax mixer was used to homogenise the broccoli and
water. Biocentrifuge was employed for centrifugation. Organic broccoli extracts were concentrated in the rotary evaporator.

Sample Preparation

Unconsumable portions (leaves and stumps) of broccoli were stripped with a knife. The edible portions (florets) were cleaned and cut into small pieces, taken apart and mixed. Pieces of broccoli florets, about 250 g, were ground with a grinder and stored under refrigeration at 4°C.

Also, microwave cooked broccoli pieces (cooking sequence 1 min. at 1400 W and 1.5 min. at 300 W) were ground in the same manner and stored again under refrigeration at 4°C.

Extraction

Sulforaphane was extracted from broccoli samples by three methods including Chiang’s method (Chiang, 1998), modified methods I and II by BFE (Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany).

Chiang’s Method

Two different samples, about 4 g of microwave cooked- and 5 g of fresh broccoli were used for sulforaphane extraction by Chiang’s method.

To each sample, 40 ml of warm distilled water (50°C) was added and thoroughly mixed by Ultra Turrax mixer at medium speed for 5 min., cooled down to 20°C and topped up to 100 g. The homogenate was centrifuged at 3500 rpm for 5 min., filtered under vacuum and 25 ml of filtrate was extracted with 25 ml of internal standard solution (stearic acid methyl ester dissolved in dichloromethane). After shaking gently for 1 min, the lower layer was dried over anhydrous sodium sulfate while being filtered by Ultrafilter. The sodium sulfate residue was washed 2-3 times with a small amount of dichloromethane.

The aqueous fraction was extracted two more times with dichloromethane as described above, concentrated to dryness under
reduced pressure at 40°C using a rotary evaporator and stored under refrigeration.

**Modified Method I by BFE**

About 5 g of fresh broccoli sample was homogenised in 40 ml of distilled water (20°C) at medium speed for 1 min using Ultra Turrax mixer. 0.1 M potassium dihydrogenphosphate solution was added to the homogenate to reduce the pH to 6, followed by 0.1 M dipotassium hydrogenphosphate for pH readjustments. All the homogenated samples were incubated in water bath shaker at 30°C for 1 h and then heated in hot water bath (80°C) for a further 7 min with occasional shaking to inhibit the myrosinase activity and cooled down to 20°C. The rest of the procedure is the same as in Chiang’s method.

**Modified Method II by BFE**

This method is the same as mentioned above in modified method I except about 4 g of microwave cooked sample was homogenised in 40 ml of distilled water (20°C) and 2.5 mg of thioglucosidase enzyme (myrosinase) was added prior to incubation.

**Determination and Optimisation of the Parameters influencing Sulforaphane Content**

In order to obtain the information on the effect of incubation temperature, the incubation temperature of all the samples (both fresh and microwave cooked) were set between 30 and 100°C respectively, while the incubation time was fixed for both the modified methods by BFE and Chiang’s method.

Alternatively, the temperature was fixed while the incubation time varied between 1 h – 18 h in modified methods for the determination of the effect of incubation time.

Variable amounts of thioglucosidase 2.5 mg – 10 mg (myrosinase) was added to microwave cooked homogenate before incubation under the
same conditions as described in modified methods for the determination of
the effect of microwave treatment and myrosinase activity.

The pressure effect on fresh samples was conducted by employing
high pressure in the range of 75 MPa - 600 MPa before homogenising in
water, but other conditions being the same as described in modified
method I.

The optimal conditions were evaluated, with respect to maximum
amount of sulforaphane collected. For the tests and analyses, two runs
were carried out and then averaged.

**Gas Chromatography Analysis**

Dried organic extract (broccoli extract) was diluted with ter-butyl
methyl ether prior to injection into GC. In GC, the carrier gas was helium
gas (pressure 1.0 bar). The initialization temperature of the column was at
50°C for 2 min, gradually raised to 250°C at 18°C min⁻¹ rate and stabilized
at 250°C for 1 min. Then the chromatograms were recorded.

**Results And Discussion**

Amount of sulforaphane, the degradation product of glucosinolates
in cruciferous vegetables such as broccoli, depends on the conditions of
hydrolysis, which includes pH, temperature, time and the pretreatment of
broccoli samples prior to storage with respect to myrosinase activity; these
parameters were studied in this research work.

Analytical results from the preliminary tests indicated that yield of
sulforaphane was higher in enzymic hydrolysis when compared to acid
hydrolysis (treatment with hydrochloric acid). The acid hydrolysis method
is less satisfactory on account of sulforaphane content.

Thermal inactivation of myrosinase from broccoli was studied at
incubation temperatures between 30 and 100°C, based on the results of
preliminary tests (Figure 1-b). The higher sulforaphane content with respect to the myrosinase activity was higher at incubation temperature of 30°C and less significant at 50°C since inactivation occurred. (Figure 1-a) These results demonstrated that myrosinase enzyme in broccoli was rather thermodabile and thermal sensitive. Ludikhuyze and Ooms (1999) reported that inactivation of myrosinase at higher temperatures may be due to limitations in heat transfer involved in the heat treatment of whole vegetables as compared to vegetable enzyme extracts and it should be noted that relatively higher temperature (80°C), is appropriate to control the enzyme activity.

The results in Figure 2 indicated that long incubation time (2 – 18 h) at the same temperature caused the lower extent of myrosinase activity in relation to the smaller amount of sulforaphane content, which is in agreement with the work done by Yen and Wei, 1993 – which also indicated that no correlation between myrosinase activity and total glucosinolate content in broccoli and other cruciferous vegetables. However, the reaction time of myrosinase and glucosinolate depends on the amount of active myrosinase and glucosinolates present in the vegetable (Howard et al., 1997; Jiao and Yu, 1998).

It could be pointed out that stable sulforaphane was formed for 1 h of incubation time and degradation of sulforaphane takes place after 1 h since sulforaphane contents decreased thereafter.

It could be observed in Figure 3 that the amount of sulforaphane, extracted from microwave cooked broccoli without adding thioglucosidase (myrosinase enzyme), were lower than those released after thioglucosidase (myrosinase) treatment. This is consistent with the prevailing concepts regarding the hydrolysis of glucosinolates by myrosinase. Sulforaphane, one of the glucosinolate break down products, was not generated to a large extent after heat treatment (blanching or microwave cooking), probably due to myrosinase inactivation (Howard et al., 1997). Clearly, myrosinase in broccoli was inactivated by heating in hot water (80°C) for a relatively
short time, 7 min. Cole (1976) and Fenwick (1989) reported that isothiocyanates are favored at pH 5 - 7 and this fact was true in the present work as notable amount of sulforaphane (isothiocyanate) were produced during enzymic hydrolysis of broccoli at pH 6.

The results in Figure 3 showed that the amount of extracted sulforaphane were nearly the same although variable amounts of thioglucosidase (myrosinase enzyme) were added, while the inactivation of natural myrosinase enzyme were being checked and also in the optimisation of the amount of myrosinase added, such that high yield of sulforaphane would be produced. The amount of thioglucosidase (myrosinase enzyme) added for activation was found to be 2.5 mg.

The preliminary literature results indicated that high hydrostatic pressure at room temperature could inactivate vegetative microorganisms and food deteriorative enzymes, while only slightly affecting nutritional and sensorial food quality aspects (Knorr, 1993). In this research work, sulforaphane content increased with increasing pressure from 75 MPa up to 600 MPa under the same conditions. Increments of sulforaphane content was higher at 25°C than that of 75°C (Figure 4).

From the analytical results, it was noted that total sulforaphane (isothiocyanate) contents vary considerably between samples of broccoli from different cultivars and the number of days after harvest (Figures 2 and 3). This may be attributed to many factors that affect the amount of glucosinolate in broccoli such as genetic, botanical and environmental effects.

Gas Chromatography method is quantitatively and qualitatively reproducible, as well as being sensitive. The sulforaphane was eluted at 9 min and was well separated from any other components in the samples (Figure 5).

Although the present work does not cover the clinical trial tests, the published datas from other scientists acknowledged that sulforaphane
definitely reduce the incidence of a number of forms of tumor in various experimental models, both in vivo in animals and in vitro in cell cultures (Zhang et al., 1994; Huang, 1994; Fahey, 1997).

Conclusion

The enzymic hydrolysis method (autolysis) led to a rapid and efficient extraction of sulforaphane. Chiang’s method, one of the enzymic hydrolysis methods, is modified by BFE (Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany). The optimum condition of incubation for maximum sulforaphane content was 1 h at 30°C and pH 6 in fresh broccoli samples. For microwave cooked broccoli samples, optimum amount of thioglucosidase (synthetic myrosinase enzyme) for activation was 2.5 mg.

Further experiments should be conducted to evaluate the optimal conditions for maximum myrosinase activity in terms of sulforaphane content in broccoli and other cruciferous vegetables of Myanmar origin, based on the same cultivar, freshly harvested from a single field in different seasons; also comparison of results of samples using different processing treatments should be made. A confirmatory clinical tests should be conducted for the anticarcinogenic functions of sulforaphane (produced under different conditions) as appropriate.

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References


Graham, S. Results of case-control studies of diets and cancer in buffalo, New York, Cancer Res. 1983, 43, 2409-2413.


Knorr, D. Effects of high-hydrostatic pressure processes on food safety and quality. Food Technol. 1993, 47(6), 156-161.


Work-up procedure for the determination of sulforaphane in braccoli

**Modified Method I**
- Mixing with water at 20°C, 1 min homogenisation by Ultra-Turrax
- Incubation at 30°C, 1 h stirring
- Inhibition the myr. act at 30°C, 7 min stirring

**Chiang's Method**
- Cleaning and Chopping the broccoli florets
- Grinding with mincer
- Mixing with water at 50°C, 5 min homogenisation by Ultra-Turrax
- Addition of water to 100 g at room temperature
- Centrifugation
- Filtration
- Extraction with CH$_2$Cl$_2$
- Evaporation of solvent
- Dissolution of the residue in TBME
- GC-Analysis

**Modified Method II**
- Microwave treatment
- Mixing with water at 20°C, 1 min homogenisation by UT (and addition of myro.)
- Incubation at 30°C, 1 h stirring
- Inhibition the myr. act at 30°C, 7 min stirring
Structure of sulforaphane
[1-isothiocyanato-4-(methyl sulfinyl)-butane]

Hydrolysis of Glucosinolates to Isothiocyanates

\[
\begin{align*}
S - C_6H_{11}O_5 & \quad + \quad H_2O \\ 
R - C & \quad \xrightarrow{\text{Thioglucosidase (myrosinase)}} \\ 
N - O - SO_3^- & \quad R - N = C = \text{isothiocyanate} \\
S & \quad C_6H_{12}O_6 \\
+ & \quad HSO_4^- \\
\text{glucose}& 
\end{align*}
\]
Figure 1(a) Influence of incubation temperature on sulforaphane content at atmospheric pressure.

Figure 1(b) Influence of temperature on myrosinase activity.
Figure 2 Influence of incubation time on sulforaphane content at atmospheric pressure.

Figure 3 Effect of myrosinase treatment on sulforaphane content in processed (microwave cooked) broccoli.
Figure 4. Effect of pressure on the sulforaphane content at different temperatures.
Figure 5 Chromatogram for the determination of sulforaphane in broccoli