Optimisation of enzymatic production of sulforaphane in broccoli sprouts and their total antioxidant activity at different growth and storage days

Ming Tian¹ · Xiaoyun Xu¹ · Hao Hu¹ · Yu Liu¹ · Siyi Pan¹

Revised: 30 November 2016 / Accepted: 16 December 2016 / Published online: 30 December 2016
© Association of Food Scientists & Technologists (India) 2016

Abstract Sulforaphane, a type of isothiocyanate hydrolysed from glucosinolate, is a powerful anticancer compound naturally found in food especially in broccoli sprouts. Despite the function of sulforaphane has been extensively studied in recent years, little attention has been given to methods that can maximize the production of this compound in broccoli sprouts. The present study optimised the enzymolysis conditions for sulforaphane production in broccoli sprouts using response surface methodology. The maximum sulforaphane production (246.95 µg/g DW) was achieved using a solid–liquid ratio of 1:30, hydrolysis time of 1.5 h, ascorbic acid content of 3.95 mg/g DW sample, and temperature of 65 °C. The highest sulforaphane content in broccoli sprouts were 233.80 µg/g DW in 5-day-old sprouts and 1555.95 µg/g DW at day 4 of storage. The highest antioxidant activities were 37.22 U/min/g DW in 3-day-old sprouts and 35.08 U/min/g DW on 4th day of storage.

Keywords Antioxidant · Broccoli sprouts · Different days · HPLC · Sulforaphane

Introduction

Broccoli sprouts are consumed globally because they contain large amounts of anticarcinogenic, antioxidant and health-promoting compounds, such as glucosinolates,

polyphenolic compounds and vitamin C (Fahey et al. 1997; Tian et al. 2016). Among these compounds, glucosinolates have attracted the most attention. The predominant glucosinolates in most broccoli varieties include glucoraphanin, glucoerucin and glucobrassicin. When broccoli sprouts are chipped, mechanically damaged or attacked by pest, glucosinolates directly bond with myrosinase and are mainly converted into isothiocyanates (ITCs), such as sulforaphane, erucin and iberin (Grubb and Abel 2006; Latté et al. 2011). Myrosinase-catalysed hydrolysis of glucosinolates initially involves cleavage of the thioglucoside linkage, yielding D-glucose and an unstable thiohydroximate-*O*-sulfonate that is spontaneously rearranged, which results in production of sulfate and a wide range of possible reaction products (Fahey et al. 2003; Angelino and Jeffery 2014). Sulforaphane (4-methylsulfinylbutyl isothiocyanate) is a naturally occurring, sulfur-containing, ITCs with excellent capacity to induce phase 2 enzymes, which are formed from glucosinolate (Nakagawa et al. 2006; Yeh and Yen 2009). Epidemiological studies show that increased consumption of sulforaphane can reduce the risk of different cancers, particularly those of the bladder, colon and lung (Cartea and Velasco 2008). Broccoli sprouts have been reported to have 20–50× the sulforaphane content of mature broccoli heads (Fahey et al. 1997).

Several studies have been conducted on the optimal enzymolysis conditions for sulforaphane production. Travers-Martin et al. (2008) pointed out that the optimal pH and temperature of myrosinase activity ranges from 4.0 to 9.0 and from 20 to 70 °C. Solid–liquid ratios have also been reported to influence the content of hydrolytic products, with the optimal proportion ranging from 1:10 to 1:40 (Powell et al. 2005; Kuang et al. 2013; Guo et al. 2014a). The optimal hydrolysis conditions for

✉ Siyi Pan
siyip@aliyun.com

¹ Key Laboratory of Environment Correlative Dietology, Ministry of Education, College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

sulforaphane in previous report as follows: temperature, 35 °C; pH, 3.0 and hydrolysis time, 4 h (Han and Row 2011). In addition, when epithelial specific protein (ESP) and Fe^{2+} bond together, large amounts of nitriles are easily formed which prevented the formation of sulforaphane (Bones and Rossiter 2006). However, EDTA acts as a metal-chelator that can decrease Fe^{2+} concentrations and block the combination of these two substances (Guo et al. 2013). Other researchers have that reaction condition of 25 °C and pH 4.0, as well as addition of 0.02 mg/g ascorbic acid are suitable for hydrolysis. An appropriate amount of ascorbic acid added to the hydrolysis solution can increase sulforaphane content, which may be attributed to the activity of a non-competitive activator of myrosinase. Excess ascorbic acid, however, could inhibit sulforaphane formation (Shikita et al. 1999; Shen et al. 2010). In the present study, the solid–liquid ratio, hydrolysis time, ascorbic acid content and temperature are shown to perform important functions in enzymatic hydrolysis. Although a large number of research works regarding optimisation of enzymatic hydrolysis conditions have been conducted, few studies have considered the combined effects of these factors. Hence, methods of maximising the sulforaphane production in broccoli sprouts are worth investigating. Meanwhile, many of the natural antioxidants exhibit a wide range of biological effects, including antiviral, antibacterial, anti-inflammatory, antithrombotic, and vasodilatory actions (Velioglu et al. 1998). Antioxidant activity is a fundamental property important for life. Many of the biological functions originate from this property, such as antimutagenicity, anticarcinogenicity, and antiaging. However, few studies have researched the total antioxidant activity of broccoli sprouts at different growth and storage days.

In the present study, the most important factors for sulforaphane production were optimised using response surface methodology (RSM). Sulforaphane in broccoli sprouts during different growth and storage days was extracted under optimal enzymolysis conditions and detected by high performance liquid chromatography (HPLC). The total antioxidant activity of the broccoli sprouts was also measured by a total antioxidant capacity (T-AOC) assay kit via the ABTS method. Results revealed the relationship of all of the important factors identified with sulforaphane production in broccoli sprouts for the first time. The model obtained can help researchers in accumulate sulforaphane for pharmaceutical and commercial use. In addition, sulforaphane content and total antioxidant activity in broccoli sprouts on different growth and storage days were compared to select the best broccoli sprouts for consumers.

Materials and methods

Plant material and cultivation condition

Broccoli seeds were obtained from XiaMen WenXing Vegetable Seed Co. First, 5 g of seeds was surface-sterilized by soaking for 2 h in 5 g/L sodium hypochlorite and then drained off. The seeds were again soaked in distilled water and then germinated under a controlled temperature of 25 °C in their natural state on a filter paper in Petri dishes. Seedlings were grown without addition of any nutrients but were spray-watered 6× per day until harvest. The sprouts were collected from the surface of pots on the 2–9 days after germination and washed with running tap water. Finally, the broccoli sprouts were lyophilized and put into 4 °C until analysis. The effect of cold storage on sulforaphane was examined by putting sprouts in –20 and 4 °C condition. The fresh samples were used to detect antioxidant activity.

Chemicals

Sulforaphane standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Tedia Company, Inc. (Ohio, USA). Analytical-grade ethyl acetate was purchased from Regent (Tianjin, China). Analytical-grade methanol and dichloromethane, as well as ascorbic acid, were all sourced from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Silica solid-phase extraction columns (200 mg/4 mL) were obtained from Alltech Associates, Inc. (Illinois, USA). All aqueous solutions were prepared with ultrapure water. Hydrochloric acid was purchased from XinYang Chemical Reagent Factory (XinYang, China). Acidic water (ultrapure water adjusted to pH 3.0 with 0.1 M hydrochloric acid) was used.

Sulforaphane extraction and purification

Extraction method was based on a previously described procedure with minor modifications (GU et al. 2012). Freeze-dried samples (0.2 g) were suspended in pH 3.0 acidic water at various solid–liquid ratios (1:20, 1:30, 1:40, 1:50 and 1:60) with various content of ascorbic acid (0, 2, 3, 4 and 5 mg/g DW). The mixtures were incubated at different temperatures (50, 55, 60, 65 and 70 °C) for various hydrolysis times (1, 2, 3, 4 and 5 h). After incubation, the mixtures were extracted with 40 mL of dichloromethane by vortexing for 15 min and then stored at room temperature for 2 h.

Purification method was based on a previously described procedure with minor modifications (Han and Row 2011).

Optimal conditions were applied on a silica SPE cartridge using ethyl acetate and methanol as the washing and eluting solvents, respectively, to eliminate interferences originating from the broccoli matrix. First, the silica SPE cartridge was conditioned with 3 mL of dichloromethane. Afterwards, the incubation mixture passed through the cartridge. This cartridge was washed with 6 mL ethyl acetate (which was discarded), and the sulforaphane was eluted with 3 mL of methanol. Finally, the methanol extract was evaporated to dryness at 42 °C for 20 min and redissolved in 2 mL of acetonitrile. The resulting solutions were vortexed for 30 s and filtered through 0.45 µm membrane. A 20 µL aliquot was injected into the HPLC system.

HPLC conditions

HPLC analyses were carried out on a Waters E2695 liquid chromatograph (Waters Corp., Milliford, MA, USA) connected to a model 2998 (DAD) photodiode array detector and controlled by Waters Empower software. Using a reversed phase C 18 column (5 µm, 250 mm × 4.6 mm; Agilent Technologies Co. Ltd., Santa Clara, USA). The solvent used was 30/70 acetonitrile/H₂O (v/v), and the flow rate was set to 0.6 mL/min. The column temperature was 36 °C. Sulforaphane was detected by spectrometer at 240 nm.

Response surface design

A three-level-four-factor Box–Behnken design (BBD) was employed after determining preliminary ranges of solid–liquid ratio (X_1), hydrolysis time (X_2), addition of ascorbic acid (X_3) and temperature (X_4) through single-factor tests. The ranges of independent variables and their levels are presented in Table 1. Sulforaphane production (Y) was considered as the response of the combination of independent variables. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where β_0 is an offset term and β_i , β_{ii} and β_{ij} are the linear, quadratic and interaction regression coefficients, respectively. All experimental design and data analyses were carried out using Design-Expert V8.0.6.1 (Trial Version, State-Ease Inc., Minneapolis, MN, USA) software to estimate the responses of the independent variables.

Antioxidant activity assay

The sample extract was prepared as described by McInerney et al. (2007) and Yuan et al. (2010) with minor

Table 1 Analytical factors and levels for RSM, and results of response surface analysis

Independent variable					Levels		
					−1	0	1
X ₁ : solid–liquid ratio					1:20	1:30	1:40
X ₂ : hydrolysis time (h)					1	2	3
X ₃ : ascorbic acid (mg)					0.6	0.8	1
X ₄ : temperature (°C)					55	60	65
No.	X ₁	X ₂	X ₃	X ₄	Sulforaphane production (µg/g DW)		
1	−1	−1	0	0	132.42 ± 4.81		
2	1	−1	0	0	142.67 ± 2.50		
3	−1	1	0	0	164.79 ± 0.94		
4	1	1	0	0	174.56 ± 4.05		
5	0	0	−1	−1	156.88 ± 1.83		
6	0	0	1	−1	150.28 ± 5.62		
7	0	0	−1	1	207.48 ± 2.99		
8	0	0	1	1	205.69 ± 2.46		
9	−1	0	0	−1	148.03 ± 5.10		
10	1	0	0	−1	164.43 ± 1.75		
11	−1	0	0	1	203.05 ± 2.12		
12	1	0	0	1	201.60 ± 1.93		
13	0	−1	−1	0	152.98 ± 6.97		
14	0	1	−1	0	163.79 ± 1.30		
15	0	−1	1	0	140.63 ± 1.69		
16	0	1	1	0	165.53 ± 1.64		
17	−1	0	−1	0	126.15 ± 2.20		
18	1	0	−1	0	178.13 ± 3.25		
19	−1	0	1	0	157.85 ± 6.67		
20	1	0	1	0	127.24 ± 2.02		
21	0	−1	0	−1	114.19 ± 0.91		
22	0	1	0	−1	218.82 ± 4.33		
23	0	−1	0	1	240.14 ± 3.19		
24	0	1	0	1	190.79 ± 2.46		
25	0	0	0	0	210.88 ± 2.48		
26	0	0	0	0	220.42 ± 2.73		
27	0	0	0	0	220.47 ± 2.54		
28	0	0	0	0	219.23 ± 4.06		
29	0	0	0	0	215.14 ± 3.07		

modifications. Duplicate samples (1 g) were extracted with 9 mL of 50% ethanol, and the sample was incubated at room temperature for 24 h in the dark. The suspension was centrifuged at 2000×*g* for 10 min at 4 °C, and the supernatants were collected for analysis. Antioxidant activity was measured using a T-AOC kit (Nanjing Jiancheng Biotech Inc., Jiangsu, China). One unit of antioxidant activity was defined as an increase in the absorbance of the reaction system by 0.01 per min per mg dry weight (DW) at 37 °C.

Statistical analysis

All data are presented as the mean $\pm$ standard deviation of three replicates. A *t* test was performed ($\alpha = 0.05$) to determine whether or not differences in mean sulforaphane content of broccoli sprouts are significant.

Results and discussion

Effect of solid–liquid ratio on sulforaphane production

Broccoli sprouts (0.2 g) were homogenised in distilled water (pH 3.0) without addition of ascorbic acid and hydrolysed at 55 °C for 3 h. As can be observed in Fig. 1a, the solid–liquid ratio significantly influenced the content of hydrolytic products ($p < 0.05$). Several reviews reported that a solid–liquid ratio between 1:10 and 1:40 is beneficial for sulforaphane formation (Powell et al. 2005; Guo et al. 2013; Kuang et al. 2013). In the present research, a solid–liquid ratio of 1:30 was found to be optimum for sulforaphane production, which may reflect

suitable conditions for myrosinase activity. Excessively high or low solid–liquid ratios were not efficient to sulforaphane formation.

Effect of hydrolysis time on sulforaphane production

Sulforaphane was produced from broccoli sprouts (0.2 g) homogenised in distilled water (pH 3.0, 8 mL) without addition of ascorbic acid and hydrolysed at 55 °C. The effect of hydrolysis time on sulforaphane production is shown in Fig. 1b. Hydrolysis time was another factor that influenced hydrolytic product content. Han and Row (2011) reported that 4 h is an optimal hydrolysis time. Recent research (Guo et al. 2013), however, identified 3 h as the best hydrolysis time. In the present study, 2 h was to be the best hydrolysis time for sulforaphane production; either shorter or longer hydrolysis times affected myrosinase activity. This result was in line with the work of Campas-Baypoli et al. (2010). Choi et al. (2004) reported considerable differences in reaction time and observed that the reaction rate appears to depend on the structure of ITCs. Lower steric hindrance of aliphatic ITCs may favor

Fig. 1 Effect of solid–liquid ratio (a), hydrolysis time (b), ascorbic acid (c), temperature (d) on sulforaphane production

Table 2 ANOVA analysis for the response variable

Variables	Sum of squares	<i>df</i>	Mean square	F value	<i>p</i> value prob > F	Significance
Model	33,883.20	14	2420.23	138.39	<0.0001	**
X ₁ : solid–liquid ratio	264.52	1	264.52	15.12	0.0016	*
X ₂ : hydrolysis time (h)	2008.55	1	2008.55	114.85	<0.0001	**
X ₃ : ascorbic acid (mg)	121.54	1	121.54	6.95	0.0195	*
X ₄ : temperature (°C)	7307.25	1	7307.25	417.83	<0.0001	**
X ₁ X ₂	0.058	1	0.058	3.294E–003	0.9550	
X ₁ X ₃	1705.28	1	1705.28	97.51	<0.0001	**
X ₁ X ₄	79.66	1	79.66	4.55	0.0510	
X ₂ X ₃	49.63	1	49.63	2.84	0.1142	
X ₂ X ₄	5927.46	1	5927.46	338.93	<0.0001	**
X ₃ X ₄	5.78	1	5.78	0.33	0.5744	
X ₁ ²	8564.77	1	8564.77	489.73	<0.0001	**
X ₂ ²	4482.74	1	4482.74	256.32	<0.0001	**
X ₃ ²	7888.65	1	7888.65	451.07	<0.0001	**
X ₄ ²	10.62	1	10.62	0.61	0.4487	
Residual	244.84	14	17.49			
Lack of fit	175.48	10	17.55	1.01	0.5431	
Pure error	69.36	4	17.34			
Cor. total	34,128.04	28				
R ² = 0.9928	R _{adj} ² = 0.9857		CV = 2.37%			

* $p < 0.05$; ** $p < 0.01$

dithiol attack of the electrophilic carbon of the N=C=S group (Liebes et al. 2001).

Effect of ascorbic acid addition on sulforaphane production

The effect of ascorbic acid on sulforaphane production mainly depended on the amount of ascorbic acid added. Contents either less or greater than the appropriate amount reduced sulforaphane production (Shen et al. 2010). Ascorbic acid is reported to function as a co-factor that promotes myrosinase activity. Ascorbic acid may thus non-competitively activate myrosinase or change its molecular structure (Shikita et al. 1999). Broccoli sprouts (0.2 g) were homogenised in distilled water (pH 3.0, 8 mL), and hydrolysed at 55 °C for 3 h. Addition of 0.8 mg (4 mg/g DW) ascorbic acid to broccoli sprouts in the present study yielded the highest content of sulforaphane (Fig. 1c). Addition of 0.2 mg of ascorbic acid to 0.2 g of broccoli sprouts was reported by Guo et al. (2013). The difference between these two results may be attributed to the diversity of broccoli sprouts.

Effect of temperature on sulforaphane production

Temperature is an important factor affecting sulforaphane production. The optimal temperature for myrosinase

activity ranged from 20 to 70 °C (Travers-Martin et al. 2008). Heating the broccoli sprouts or florets to 60 °C could significantly decrease ESP activity and thus increase sulforaphane formation (Pérez et al. 2014). Broccoli sprouts (0.2 g) were homogenised in pH 3.0 distilled water (8 mL), and hydrolysed for 3 h. Figure 1d shows that temperature between 50 and 65 °C affect sulforaphane formation; here, the optimum temperature appeared to be 60 °C. This result may be explained by the fact that both solubility and mass transfer rate increase with temperature. Temperature-induced increases in mass transfer rates promote rapid equilibrium (Powell et al. 2005).

Statistical analysis and the model fitting

BBD with a total of 29 runs, four factors and three levels was used to fit a second-order response surface and optimise the extraction conditions. The process variables and experimental data are shown in Table 1, while the results of analysis of variance, goodness-of-fit and the adequacy of the models are shown in Table 2. The maximum value (240.14 ± 3.19 µg/g DW) of sulforaphane production was observed at a solid–liquid ratio of 1:30, hydrolysis time of 1 h, ascorbic acid content of 0.8 mg and temperature of 65 °C. The parameters of the equation were obtained by multiple regression analysis of the experimental data. The

following polynomial Eq. (2) explains the experimental data

$$\begin{aligned}
 Y = & 217.23 + 4.7X_1 + 12.94X_2 - 3.18X_3 + 24.68X_4 \\
 & - 0.12X_1X_2 - 20.65X_1X_3 - 4.46X_1X_4 + 3.52X_2X_3 \\
 & - 38.49X_2X_4 + 1.2X_3X_4 - 36.34X_1^2 - 26.29X_2^2 \\
 & - 34.87X_3^2 - 1.28X_4^2.
 \end{aligned}
 \tag{2}$$

The coefficient $R^2 = 0.9928$ indicated that 99.28% of the total variation can be explained by the model equation. The value of the adjusted coefficient of determination ($R_{adj}^2 = 0.9857$) showed that the model is significant. The model p value (prob > F) was <0.0001, which also suggests that the model is highly significant. The F-value for the lack of fit was insignificant ($p > 0.05$), which confirms the validity of the model. As well, the coefficient of variation of the model (CV = 2.37%) indicated considerably high degrees of precision and reliability of the experiment values.

The linear effects of solid–liquid ratio, hydrolysis time, ascorbic acid content and temperature were all significant ($p < 0.05$) for sulforaphane production (Table 2). Interactive terms between solid–liquid ratio and ascorbic acid content ($p < 0.05$), as well as between hydrolysis time and temperature ($p < 0.05$) were significant for sulforaphane production. The quadratic term of solid–liquid ratio, hydrolysis time and ascorbic acid were significant ($p < 0.01$). These results indicate that both hydrolysis time and temperature perform dominant functions during sulforaphane production.

Optimisation of glucosinate enzymolysis conditions for sulforaphane production

The results of sulforaphane production influenced by the interaction of variables are presented in Fig. 2. Response surface plots were constructed by plotting two independent variables while two other variables were fixed at zero level. The 3-D response surface and contour plots in Fig. 2a (ascorbic acid and temperature at zero levels) show that sulforaphane production initially increases and then gradually decreases as the solid–liquid ratio increased from 1:20 to 1:40. The same trend was observed when hydrolysis time was increased from 1 to 3 h. In Fig. 2b, hydrolysis time and temperature were fixed at zero levels. Results obtained show that sulforaphane production initially increases and then gradually decreases as the solid–liquid ratios increased from 1:20 to 1:40 and ascorbic acid addition increased from 0.6 (3) to 1.0 mg (5 mg/g DW). Under a fixed hydrolysis time and ascorbic acid at zero levels (Fig. 2c), sulforaphane production initially increased and then gradually decreased when as the solid–liquid ratio increased from 1:20 to 1:40; this trend also increased as

temperature increased from 55 to 65 °C. The 3-D and contour plots in Fig. 2d, wherein the solid–liquid and temperature were set to zero levels, show that sulforaphane production increases initially and then decreases gradually as the hydrolysis time increases from 1 to 3 h; the same trend was observed as ascorbic acid addition increased from 0.6 (3) to 1.0 mg (5 mg/g DW). In Fig. 2e, sulforaphane production initially increased and then gradually decreased as hydrolysis time increased from 1 to 3 h. Production also invariably increased as temperature increased from 55 to 65 °C. These results are further confirmed in Fig. 2f, which shows solid–liquid ratio and hydrolysis time fixed at zero levels, sulforaphane production initially increased and then gradually decreased as ascorbic acid addition increased from 0.6 (3) to 1.0 mg (5 mg/g DW); production also consistently increased when temperature was increased from 55 to 65 °C.

According to the single-factor study and analysis of Fig. 2, the optimal enzymolysis conditions for sulforaphane production are as follows: solid–liquid ratio of 1:30.21, hydrolysis time of 1.51 h, ascorbic acid content of 3.95 mg/g DW, and temperature of 65 °C. Under these conditions, the predicted maximum sulforaphane amount produced is 246.95 µg/g DW.

Model verification

Verification of the model equation was tested by using the selected optimal conditions. The maximum content of sulforaphane was 253.58 ± 0.72 µg/g DW. This result agree with the value predicted by the model, and no significant differences ($p > 0.05$) were observed. These findings suggest that the model is valid and practical.

Change in sulforaphane contents during different growth and storage days

After irrigation, sulforaphan contents, which ranged between 3.80 and 233.80 µg/g DW, evidently changed during growth days 2–9 (Fig. 3a). Sulforaphane contents remained higher in 2–5-day-old sprouts than in sprouts of other days; in fact, maximum values were observed on day 5 (233.80 µg/g DW). Similar results were reported by Fahey, who found that 3-day-old sprouts contain maximum amount of sulforaphane (20-fold compared with that in mature broccoli) (Fahey et al. 1997). Previous research has shown that broccoli sprouts exhibit decreased sulforaphane production during the first 3–7 days (Pérez-Balibrea et al. 2008). Glucoraphanin contents and sulforaphane formation also decline with sprout growth (Guo et al. 2014b). Sulforaphane is a sulfur-containing isothiocyanates that is hydrolyzed from glucosinolates by myrosinase. Sulforaphane production decreased with seed growth because

Fig. 2 Response surface plots show the interactions of variable on sulforaphane production.

a Interaction between solid-liquid ratio and hydrolysis time.
b Interaction between solid-liquid ratio and ascorbic acid.
c Interaction between solid-liquid ratio and temperature.
d Interaction between hydrolysis time and ascorbic acid.
e Interaction between hydrolysis time and temperature.
f Interaction between ascorbic acid and temperature

Fig. 3 The sulforaphane production during different growth days, storage condition and storage days

of degradation of glucosinolates to support the formation of other sulfur-containing compounds, particularly under low-sulfur conditions (Falk et al. 2007). Much of the reduction in concentration may be simply a result of plant expansion, which diluted the compounds and decreased their content per weight unit (Doughty et al. 1991). Therefore, the above results were reasonable.

The effect of cold storage on sulforaphane concentration was examined by placing 5-day-old broccoli sprouts into -20 and 4 °C conditions for 1 week. Equal-quality broccoli sprouts were weighed daily for a week and collected the sprouts which in the same storage condition together. The results in Fig. 3b show that freezing (-20 °C, 1 week) was more favourable for retaining sulforaphane than refrigeration (4 °C, 1 week). Using the 5-day-old broccoli sprouts as frozen materials, the trend of sulforaphane content (Fig. 3c) was observed to initially increase and then sharply decrease during different storage days. Broccoli sprouts stored for 4 D retained maximum sulforaphane contents nearly 1555.95 µg/g DW. Previous studies have shown that low temperatures result in longer shelf lives than room temperatures because the former promotes better color appearance and lower respiration during storage. Most of the researchers just studied the temperature above 0 °C, depends on the advantage of both visual quality and glucosinolate content (Jones et al. 2006). However,

glucosinolate contents in broccoli are best maintained by freezing (Rodrigues and Rosa 1999). At temperatures below 0 °C (-20 °C, which can be easily implemented at home), sulforaphane production increases because of glucosinolates in vacuoles and myrosinase attached to the intracellular membrane. When broccoli sprouts freezes and expands, the plant tissue is broken down and glucosinolate-myrosinase system can contact easily, forming an abundance of sulforaphane (Rosa et al. 1997). Meanwhile, when plant growth condition changed sharply, it can activate glucosinolate-myrosinase system, thus affecting the content of sulforaphane (GU et al. 2012). However, the decline in sulforaphane content also may due to degradation of glucosinolate and dilution of plant expansion (Doughty et al. 1991; Falk et al. 2007).

Hence, fresh broccoli sprouts possess high sulforaphane contents during early germination and maintain high levels of the compound when briefly stored at -20 °C.

Change in total antioxidant activity during different growth and storage days

The total antioxidant activity of broccoli sprouts during different growth and storage days are shown in Fig. 4a, b, respectively. The total trend of antioxidant activity was increased on the first day and then declined during different

Fig. 4 The antioxidant activity of broccoli sprouts during different growth and storage days

growth days. During storage, antioxidant activity initially declined, increased sharply on the third day, and then continued to decrease thereafter. Previous studies have reported that the antioxidant activity of radish sprouts gradually decreased from days 3–7. The sulforaphane content and total antioxidant activity did not show significant correlation. Similar results were reported by Yuan et al. (2010). Sulforaphane is not a direct-acting antioxidant because the isothiocyanate group hardly participates in oxidation or reduction reactions under physiological conditions. Numerous factors, such as phenolic compounds, vitamin C, glucosinolates and so on, could affect antioxidant activity. A significant positive correlation between antioxidant activity and phenolic compounds of radish sprouts has been reported (Kim et al. 2006), and low temperature (-20°C) have been observed to contributed to higher total antioxidant activity, as reported by Mahn et al. (2012) Total polyphenols contents are only slightly affected by freeze-drying conditions. In our previous research, young broccoli sprouts contain lots of health-promoting compounds, such as phenolic, Anthocyanin and Flavonoids (Tian et al. 2016). All these compounds may contribute to their antioxidant activity. In summary, broccoli sprouts in the early growth and middle storage days during a week contain high levels of antioxidant compounds. This result could also be brought by different genotype, we will verify the hypothesis in the future.

Conclusion

In the present study, several factors, including solid–liquid ratio, hydrolysis time, ascorbic acid addition and temperature, presented significant effects on sulforaphane production from glucosinolate. This study is the first to analyse the interactive relationship among the four factors,

described above; in particular, we found that the interactive terms between solid and liquid ratio and ascorbic acid, as well as those between hydrolysis time and temperature, are significant for sulforaphane production. The results of sulforaphane content and total antioxidant activity in broccoli sprouts during different growth and storage days showed that broccoli sprouts in early growth and middle storage days during a week contain high levels of antioxidant compounds. The model obtained by RSM as well as new results can help researchers accumulate sulforaphan for pharmaceutical and commercial use.

References

- Angelino D, Jeffery E (2014) Glucosinolate hydrolysis and bioavailability of resulting isothiocyanates: focus on glucoraphanin. *J Funct Foods* 7:67–76. doi:10.1016/j.jff.2013.09.029
- Bones AM, Rossiter JT (2006) The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry* 67:1053–1067. doi:10.1016/j.phytochem.2006.02.024
- Campas-Baypoli ON, Sánchez-Machado DI, Bueno-Solano C et al (2010) HPLC method validation for measurement of sulforaphane level in broccoli by-products. *Biomed Chromatogr* 24:387–392. doi:10.1002/bmc.1303
- Cartea ME, Velasco P (2008) Glucosinolates in Brassica foods: bioavailability in food and significance for human health. *Phytochem Rev* 7:213–229. doi:10.1007/s11101-007-9072-2
- Choi MMF, Shuang S, Lai HY et al (2004) Gas chromatography-mass spectrometric determination of total isothiocyanates in Chinese medicinal herbs. *Anal Chim Acta* 516:155–163. doi:10.1016/j.aca.2004.04.010
- Doughty KJ, Porter AJR, Morton AM et al (1991) Variation in the glucosinolate content of oilseed rape (*Brassica napus* L.) leaves. *Ann Appl Biol* 118:469–477. doi:10.1111/j.1744-7348.1991.tb05648.x
- Fahey JW, Zhang Y, Talalay P (1997) Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci USA* 94:10367–10372. doi:10.1073/pnas.94.19.10367

- Fahey JW, Wade KL, Stephenson KK, Chou FE (2003) Separation and purification of glucosinolates from crude plant homogenates by high-speed counter-current chromatography. *J Chromatogr A* 996:85–93. doi:[10.1016/S0021-9673\(03\)00607-1](https://doi.org/10.1016/S0021-9673(03)00607-1)
- Falk KL, Tokuhisa JG, Gershenzon J (2007) The effect of sulfur nutrition on plant glucosinolate content: physiology and molecular mechanisms. *Plant Biol* 9:573–581. doi:[10.1055/s-2007-965431](https://doi.org/10.1055/s-2007-965431)
- Grubb CD, Abel S (2006) Glucosinolate metabolism and its control. *Trends Plant Sci* 11:89–100. doi:[10.1016/j.tplants.2005.12.006](https://doi.org/10.1016/j.tplants.2005.12.006)
- Gu ZX, Guo QH, Gu YJ (2012) Factors influencing glucoraphanin and sulforaphane formation in brassica plants: a review. *J Integr Agric* 11:1804–1816. doi:[10.1016/S2095-3119\(12\)60185-3](https://doi.org/10.1016/S2095-3119(12)60185-3)
- Guo Q, Guo L, Wang Z et al (2013) Response surface optimization and identification of isothiocyanates produced from broccoli sprouts. *Food Chem* 141:1580–1586. doi:[10.1016/j.foodchem.2013.04.026](https://doi.org/10.1016/j.foodchem.2013.04.026)
- Guo L, Yang R, Wang Z et al (2014a) Effect of NaCl stress on health-promoting compounds and antioxidant activity in the sprouts of three broccoli cultivars. *Int J Food Sci Nutr* 65:476–481. doi:[10.3109/09637486.2013.860583](https://doi.org/10.3109/09637486.2013.860583)
- Guo L, Yang R, Wang Z et al (2014b) Glucoraphanin, sulforaphane and myrosinase activity in germinating broccoli sprouts as affected by growth temperature and plant organs. *J Funct Foods* 9:70–77. doi:[10.1016/j.jff.2014.04.015](https://doi.org/10.1016/j.jff.2014.04.015)
- Han D, Row KH (2011) Separation and purification of sulforaphane from broccoli by solid phase extraction. *Int J Mol Sci* 12:1854–1861. doi:[10.3390/ijms12031854](https://doi.org/10.3390/ijms12031854)
- Jones RB, Faragher JD, Winkler S (2006) A review of the influence of postharvest treatments on quality and glucosinolate content in broccoli (*Brassica oleracea* var. *italica*) heads. *Postharvest Biol Technol* 41:1–8. doi:[10.1016/j.postharvbio.2006.03.003](https://doi.org/10.1016/j.postharvbio.2006.03.003)
- Kim HJ, Chen F, Wang X, Choi JH (2006) Effect of methyl jasmonate on phenolics, isothiocyanate, and metabolic enzymes in radish sprout (*Raphanus sativus* L.). *J Agric Food Chem* 54:7263–7269. doi:[10.1021/jf060568c](https://doi.org/10.1021/jf060568c)
- Kuang P, Song D, Yuan Q et al (2013) Separation and purification of sulforaphane from radish seeds using macroporous resin and preparative high-performance liquid chromatography. *Food Chem* 136:342–347. doi:[10.1016/j.foodchem.2012.08.082](https://doi.org/10.1016/j.foodchem.2012.08.082)
- Latté KP, Appel KE, Lampen A (2011) Health benefits and possible risks of broccoli—an overview. *Food Chem Toxicol* 49:3287–3309. doi:[10.1016/j.fct.2011.08.019](https://doi.org/10.1016/j.fct.2011.08.019)
- Liebes L, Conaway CC, Hochster H et al (2001) High-performance liquid chromatography-based determination of total isothiocyanate levels in human plasma: application to studies with 2-phenethyl isothiocyanate. *Anal Biochem* 291:279–289. doi:[10.1006/abio.2001.5030](https://doi.org/10.1006/abio.2001.5030)
- Mahn A, Zamorano M, Barrientos H, Reyes A (2012) Optimization of a process to obtain selenium-enriched freeze-dried broccoli with high antioxidant properties. *LWT Food Sci Technol* 47:267–273. doi:[10.1016/j.lwt.2012.01.017](https://doi.org/10.1016/j.lwt.2012.01.017)
- McInerney JK, Seccafien CA, Stewart CM, Bird AR (2007) Effects of high pressure processing on antioxidant activity, and total carotenoid content and availability, in vegetables. *Innov Food Sci Emerg Technol* 8:543–548. doi:[10.1016/j.ifset.2007.04.005](https://doi.org/10.1016/j.ifset.2007.04.005)
- Nakagawa K, Umeda T, Higuchi O et al (2006) Evaporative light-scattering analysis of sulforaphane in broccoli samples: quality of broccoli products regarding sulforaphane contents. *J Agric Food Chem* 54:2479–2483. doi:[10.1021/jf051823g](https://doi.org/10.1021/jf051823g)
- Pérez C, Barrientos H, Román J, Mahn A (2014) Optimization of a blanching step to maximize sulforaphane synthesis in broccoli florets. *Food Chem* 145:264–271. doi:[10.1016/j.foodchem.2013.08.053](https://doi.org/10.1016/j.foodchem.2013.08.053)
- Pérez-Balibrea S, Moreno DA, García-Viguera C (2008) Influence of light on health-promoting phytochemicals of broccoli sprouts. *J Sci Food Agric* 88:904–910. doi:[10.1002/jsfa](https://doi.org/10.1002/jsfa)
- Powell EE, Hill GA, Juurlink BHJ, Carrier DJ (2005) Glucoraphanin extraction from *Cardaria draba*: part 1. Optimization of batch extraction. *J Chem Technol Biotechnol* 80:985–991. doi:[10.1002/jctb.1273](https://doi.org/10.1002/jctb.1273)
- Rodrigues AS, Rosa EAS (1999) Effect of post-harvest treatments on the level of glucosinolates in broccoli. *J Sci Food Agric* 79:1028–1032. doi:[10.1002/\(SICI\)1097-0010\(19990515\)79:7<1028::AID-JSFA322>3.0.CO;2-I](https://doi.org/10.1002/(SICI)1097-0010(19990515)79:7<1028::AID-JSFA322>3.0.CO;2-I)
- Rosa EAS, Heaney RK, Fenwick GR, Portas CAM (1997) Glucosinolates in crop plants. *J Hortic Rev* 19:99–215. doi:[10.1002/9780470650622.ch3](https://doi.org/10.1002/9780470650622.ch3)
- Shen L, Su G, Wang X et al (2010) Endogenous and exogenous enzymolysis of vegetable-sourced glucosinolates and influencing factors. *Food Chem* 119:987–994. doi:[10.1016/j.foodchem.2009.08.003](https://doi.org/10.1016/j.foodchem.2009.08.003)
- Shikita M, Fahey JW, Golden TR et al (1999) An unusual case of “uncompetitive activation” by ascorbic acid: purification and kinetic properties of a myrosinase from *Raphanus sativus* seedlings. *Biochem J* 341(Pt 3):725–732. doi:[10.1042/0264-6021:3410725](https://doi.org/10.1042/0264-6021:3410725)
- Tian M, Xu X, Liu Y et al (2016) Effect of Se treatment on glucosinolate metabolism and health-promoting compounds in the broccoli sprouts of three cultivars. *Food Chem* 190:374–380. doi:[10.1016/j.foodchem.2015.05.098](https://doi.org/10.1016/j.foodchem.2015.05.098)
- Travers-Martin N, Kuhlmann F, Müller C (2008) Revised determination of free and complexed myrosinase activities in plant extracts. *Plant Physiol Biochem* 46:506–516. doi:[10.1016/j.plaphy.2008.02.008](https://doi.org/10.1016/j.plaphy.2008.02.008)
- Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J Agric Food Chem* 46:4113–4117. doi:[10.1021/jf9801973](https://doi.org/10.1021/jf9801973)
- Yeh CT, Yen GC (2009) Chemopreventive functions of sulforaphane: a potent inducer of antioxidant enzymes and apoptosis. *J Funct Foods* 1:23–32. doi:[10.1016/j.jff.2008.09.002](https://doi.org/10.1016/j.jff.2008.09.002)
- Yuan G, Wang X, Guo R, Wang Q (2010) Effect of salt stress on phenolic compounds, glucosinolates, myrosinase and antioxidant activity in radish sprouts. *Food Chem* 121:1014–1019. doi:[10.1016/j.foodchem.2010.01.040](https://doi.org/10.1016/j.foodchem.2010.01.040)