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Response surface optimization and identification of isothiocyanates produced from broccoli sprouts

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ABSTRACT

Isothiocyanates (ITCs) are proved as one of natural anticarcinogenic compounds, which are produced from the decomposition of glucosinolates by myrosinase. The present study optimized the enzymolysis conditions (pH, addition of EDTA and ascorbic acid) for ITCs production from glucosinolates in broccoli sprouts using response surface methodology. ITCs production was clearly enhanced by a suitable pH, addition content of EDTA and ascorbic acid. The optimal enzymolysis conditions were determined to be adding EDTA 0.02 mmol and 0.16 mg ascorbic acid to 4 ml of the homogenized phosphate–citrate buffer solution (pH 4.00). ITCs profiles were identified and seven kinds of individual ITCs were detected, among which sulforaphane accounted the most. Four kinds of individual ITCs including isobutyl isothiocyanate, 4-isothiocyanato-1-butene, 1-isothiocyanato-3-methyl-butane and 1-isothiocyanato-butane are firstly reported in broccoli sprouts.

1. Introduction

Dietary intake of *Brassica* vegetables (e.g., broccoli, cabbage, cauliflower, etc.) can reduce the risks of chronic diseases such as atherosclerosis and cancer (Herr & Büchler, 2010). These beneficial effects are mainly attributed to glucosinolates, a group of thioglucosides that are compounds of an activated chemical defense (Verkerk et al., 2009). Upon disruption of plant tissues and cells, glucosinolates can be hydrolyzed by myrosinase into isothiocyanates (ITCs), nitriles, thiocyanates, epithionitriles and oxazolidines (Bones & Rossiter, 2006). ITCs have been proved as one of natural anticarcinogenic compounds (Cartea & Velasco, 2007).

Broccoli (*Brassica oleracea* L. var. *italics* Plenck) is rich in antioxidants, vitamin C and health-promoting compounds such as glucosinolates, phenolics and anthocyanins (Jeffery et al., 2003). However, it is the glucosinolates that attracts the highest attention. The predominant glucosinolates in most broccoli varieties are glucoraphanin, glucoerucin and glucobrassicin (Guo, Yuan, & Wang, 2011; Latté, Appel, & Lampen, 2011), whose enzymolytic ITCs are sulforaphane, erucin and iberin, respectively. Sulforaphane has been reported to be a naturally inducer of phase II detoxication enzymes to detoxify cancer-causing chemicals (Fahey, Zhang, & Talalay, 1997). Erucin has presented promising anticancer effects in many *in vitro* and *in vivo* studies (Melchini & Traka, 2010). In addition, a metabolic interconversion of sulforaphane to erucin in humans was found because they are structurally related (Melchini & Traka, 2010). Iberin could inhibit the growth and induce apoptosis in human glioblastoma cells (Jadhav, Ezhilarasan, Vaughn, Berhow, & Mohanam, 2007). Hence, how to enlarge the production of enzymolytic ITCs from broccoli worth investigating.

Broccoli sprouts obtained from seed germination are richer in glucosinolates than their mature counterparts. There are 20 times more glucoraphanin in broccoli sprouts compared with the mature broccoli (Fahey et al., 1997). Significant physiological and biochemical metabolisms occur during broccoli seed germination (Gu, Guo, Zhang et al., 2012). Being a co-factor that enhances myrosinase activity, the ascorbic acid in broccoli sprouts accumulated with germination time (Pérez-Balibrea, Moreno, & García-Viguera, 2011) and myrosinase activity was the highest at 2 day-old sprouts (Williams, Critchley, Pun, Nottingham, & O'Hare, 2008). The above issues suggest that broccoli sprouts are more promising for ITCs production than mature ones.

The production of enzymolytic products from broccoli sprouts relies on the enzymolysis conditions, including temperature, reaction time, pH, the presence of Fe^{2+} and epithiospecifier protein (ESP) (Gu, Guo, & Gu, 2012). It is commonly believed that at pH 6–7, the generally products are ITCs, while under the presence of ESP and Fe^{2+} or in an acidic condition, large amounts of nitriles are formed (Latté et al., 2011). However, Shen et al. (Shen, Su, Wang, Du, & Wang, 2010) found that the highest conversion rate of glucosinolates to sulforaphane was at pH 4.0. Hence, whether a neutral condition gives the most ITCs production needs further confirmation. ESP needs Fe^{2+} to activate, suggesting that decreas-

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ing the Fe^{2+} content in broccoli sprouts through adding EDTA exhibits the potential of inhibiting nirtiles formation and thus enhancing ITCs production. In addition, ascorbic acid were reported to be able to stimulate myrosinase activity and thus improve the hydrolysis of glucosinolates (Gu, Guo, & Gu, 2012). To the most of our knowledge, no information is available on factors influencing ITCs production from enzymolytic glucosinolates in broccoli sprouts as well as the optimization of this enzymolysis conditions.

In the present study, the enzymolysis conditions (pH, addition of EDTA and ascorbic acid) for ITCs production were optimized using response surface methodology (RSM). Then the ITCs profiles produced from broccoli sprouts using the optimal enzymolysis conditions were identified by GC/MS. The results would for the first time bring a better understanding of the combined effects of the above key processing variables on ITCs production from broccoli sprouts. The obtained model would be useful for researchers working on chemopreventive properties of *Brassica* plants to accumulate more ITCs for pharmaceutical use. In addition, the individual ITCs identified could provide a chance to investigate more on other individual ITC except for sulforaphane in broccoli sprouts.

2. Materials and methods

2.1. Materials and reagents

Seeds of broccoli (*B. oleracea* var. *italica*, cv. Lvlingxiang) were purchased from Nanjing Jinshengda Seed Co. Ltd. (Jiangsu, China). Standard sample of sulforaphane was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade and purchased from Shanghai Institute of Biochemistry (Shanghai, China).

2.2. Seed germination

Dry seeds were immersed in 1.5% sodium hypochlorite for 15 min, then drained and washed with distilled water until they reached neutral pH. They were then placed in distilled water and soaked at 30 °C for 4 h. Soaked seeds were then germinated on a filter paper in petri dishes (15 cm in diameter) filled with sterilized quartz sand and grew in the incubators at 30 °C in darkness for 4 days. Seeds in each petri dish were supplied the sowing day with 60 ml distilled water and every 12 h with 20 ml of 4 mmol/L $ZnSO_4$. Finally, 4-day-old sprouts were collected for measurements. Sprout samples were rapidly and gently collected from the surface of the filter paper. Then the fresh weight (FW) of the broccoli sprouts was weighed, after which they were immediately frozen in liquid nitrogen and kept in polyethylene bags at −80 °C for further experiments.

2.3. Release of ITCs from glucosinolates

Our previous study before the RSM experiment has obtained the enzymolysis time (3 h) and temperature (40 °C) for completely release of ITCs from glucosinolates. This was used for the following experiments.

2.4. Extraction and determination of ITCs production

Briefly, fresh broccoli sprouts (0.2 g) were homogenized in 4 ml of phosphate–citrate buffer solution at various pH (pH 3.0, 4.0, 5.0, 6.0, and 7.0) with the various addition content of EDTA (0, 0.02, 0.04, 0.06, and 0.08 mmol) and ascorbic acid (0, 0.10, 0.20, 0.30, and 0.40 mg). The mixture was incubated at 40 °C for 3 h to completely release ITCs from glucosinolates. After incubation, 3 ml of methylene dichloride was added and extracted for 30 min. The mixture was then centrifuged at 10,000 rpm for 15 min and the fraction containing ITCs was carefully collected. The reaction solution consisting of 2 ml of methanol, 1.8 ml of 50 mmol/L sodium borate buffer (pH 8.5), 0.2 ml of 7 mmol/L 1,2-benzenedithiol and 1 ml of the fraction were incubated at 65 °C for 1 h. The produced ITCs content was determined by quantifying the reacted cyclic product of ITCs and 1,2-benzenedithiol using HPLC and calculated from sulforaphane standard curve (Jiao, Yu, Hankin, Low, & Chung, 1998). Content of ITCs produced was expressed as milligrams per 100 g FW.

HPLC conditions: A Waters μBondapak C_{18} (150 × 3.9 mm) with a C_{18} Waters μBondapak guard column was used. The mobile phase consisted of 70% methanol and 30% H_2O at a flow rate of 1.75 ml/min with a sample injection volume of 20 μl. The detection wavelength was set at 365 nm.

2.5. Experimental design

After determining the preliminary range of pH, addition of EDTA and ascorbic acid through single-factor test, a three-level-three-factor, Box–Behnken design (BBD) was employed in this optimization study. The pH value (X_1), addition content of EDTA (X_2) and ascorbic acid (X_3) were the independent variables selected to be optimized for enzymolysis conditions for ITCs production. The range of independent variables and their levels were presented in Table 1. ITCs production (Y) was taken as the response for the combination of the independent variables. All the experiments were conducted randomly to minimize the effect of unexplained variability in the observed responses due to systematic errors. 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 Y is the dependent variables. β_0 is an offset term. β_i, β_{ii}, β_{ij} are the linear, quadratic, and interaction regression coefficients, respectively and X_i and X_j are levels of the independent variables. Analysis of the experimental design and calculation of predicted data were carried out by Design-Expert 8.0.5 (Trial Version, State-Ease Inc.,

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

Independent variables			Levels		
			−1	0	1
X_1: pH			4.00	5.00	6.00
X_2: EDTA (mmol)			0.02	0.04	0.06
X_3: Ascorbic acid (mg)			0.10	0.20	0.30
No.	X_1 pH	X_2 EDTA (mmol)	X_3 Ascorbic acid (mg)		Y: ITCs production (mg/100 g FW)
1	−1	−1	0		690.84 ± 22.43
2	1	−1	0		584.84 ± 19.90
3	−1	1	0		674.73 ± 16.62
4	1	1	0		590.60 ± 13.97
5	−1	0	−1		642.63 ± 21.89
6	1	0	−1		541.23 ± 13.08
7	−1	0	1		597.62 ± 15.26
8	1	0	1		535.48 ± 10.10
9	0	−1	−1		614.89 ± 20.90
10	0	1	−1		566.55 ± 15.72
11	0	−1	1		560.80 ± 18.04
12	0	1	1		569.88 ± 16.13
13	0	0	0		618.34 ± 17.39
14	0	0	0		609.45 ± 19.66
15	0	0	0		604.78 ± 13.12
16	0	0	0		610.07 ± 11.22
17	0	0	0		598.82 ± 14.49