J Food Sci Technol (November 2016) 53(11):4110–4115
DOI 10.1007/s13197-016-2386-6

SHORT COMMUNICATION

Optimization of an incubation step to maximize sulforaphane content in pre-processed broccoli

Andrea Mahn[1] · **Carmen Pérez**[2]

Revised: 11 October 2016 / Accepted: 20 October 2016 / Published online: 9 November 2016

Abstract Sulforaphane is a powerful anticancer compound, found naturally in food, which comes from the hydrolysis of glucoraphanin, the main glucosinolate of broccoli. The aim of this work was to maximize sulforaphane content in broccoli by designing an incubation step after subjecting broccoli pieces to an optimized blanching step. Incubation was optimized through a Box–Behnken design using ascorbic acid concentration, incubation temperature and incubation time as factors. The optimal incubation conditions were 38 °C for 3 h and 0.22 mg ascorbic acid per g fresh broccoli. The maximum sulforaphane concentration predicted by the model was 8.0 μmol g^{-1}, which was confirmed experimentally yielding a value of 8.1 ± 0.3 μmol g^{-1}. This represents a 585% increase with respect to fresh broccoli and a 119% increase in relation to blanched broccoli, equivalent to a conversion of 94% of glucoraphanin. The process proposed here allows maximizing sulforaphane content, thus avoiding artificial chemical synthesis. The compound could probably be isolated from broccoli, and may find application as nutraceutical or functional ingredient.

Keywords Broccoli · Incubation · Sulforaphane · Optimization

✉ Andrea Mahn
andrea.mahn@usach.cl

[1] Department of Chemical Engineering, University of Santiago of Chile, Avenida Libertador Bernardo O'Higgins 3363, 9170019 Estación Central, Santiago, Chile

[2] Present Address: Department of Agroindustrial Engineering, Pontificia Bolivariana University, Cra. 6 No. 97A-99, Montería, Colombia

Introduction

Sulforaphane is considered as the most powerful anticancer compound found naturally in food (Elbarbry and Elrody 2011). Besides, it acts as indirect antioxidant at cellular level by inducing nuclear factor (erythoid-derived 2)—like 2 (Nrf2) and stimulating the synthesis of antioxidant enzymes (Zhou et al. 2016). Its precursor is glucoraphanin, which is the most abundant glucosinolate in some broccoli cultivars. Glucoraphanin has no bioactive effect when ingested. The hydrolysis of glucoraphanin to yield sulforaphane proceeds through the action of myrosinase (EC 3.2.1.147), which is found in *Brassicaceae* plants as well as in the intestine microbiota (Shapiro et al. 2001). Unfortunately, the chemical conditions in the intestine disfavor sulforaphane formation since the hydrolysis privileges the formation of other compounds. Then, the bioavailability of sulforaphane can be improved if it is ingested directly in its active form.

In the intact plant tissue, glucoraphanin is physically segregated from the enzyme. When the vegetal tissue is disrupted through chewing or processing, glucoraphanin interacts with myrosinase and the hydrolysis proceeds, yielding sulforaphane as well as other compounds such as nitriles, thiocyanates and epithionitriles. The formation of these products is determined by the chemical conditions, i.e. pH, temperature, presence of Fe^{+2}, presence and activity of epithiospecifier protein (ESP) (Gu et al. 2012) and the concentration of ascorbic acid, which acts as a co-factor of myrosinase (Bones and Rossiter 2006). Hence, selecting the processing conditions of broccoli that favor the formation of sulforaphane seems attractive for developing a broccoli-based functional food, naturally enriched in the anticancer compound sulforaphane.

Some authors investigated broccoli processing under different conditions in order to favor sulforaphane

formation and at the same time minimize the formation of nitriles and other products (van Eylen et al. 2009; Jones et al. 2010; Wang et al. 2012). Specific time–temperature combinations in hydrothermal treatments of broccoli increase the sulforaphane content (Matusheski et al. 2004; Wang et al. 2012), most likely due to denaturation of ESP at temperatures above 40 °C. Matusheski et al. (2004) reported that heating broccoli at 60 °C during 5 or 10 min produced an increase in sulforaphane content. Guo et al. (2013) maximized the formation of isothiocyanates in broccoli sprouts by incubation at 40 °C during 4 h in 4 mL citrate–phosphate buffer pH 4.0 containing 0.02 mmol EDTA and 0.16 mg ascorbic acid. Recently, Pérez et al. (2014) reported that sulforaphane content of broccoli was maximized when subjecting the vegetable to a blanching step consisting of immersion in water at 57 °C during 13 min. In that work, the authors also showed that despite increasing 3.3-fold the sulforaphane content with respect to the fresh vegetable, there was still a considerable amount of glucoraphanin (approximately 8 μmol g^{-1} dry weight) that was not hydrolyzed. Then, incubating the blanched broccoli under optimal conditions would allow the complete conversion of glucoraphanin and hence the maximum sulforaphane formation.

The aim of this work was to determine the incubation conditions that maximize the content of sulforaphane in pre-processed broccoli. The effect of processing conditions on sulforaphane content and myrosinase activity was also investigated.

Materials and methods

Plant material

Broccoli (*Brassica oleracea* var *Italica* cv. Avenger) heads (3 days after harvesting) were purchased at the local market (Santiago, Chile) from a single supplier. Broccoli heads were washed and cut into single florets of 5-cm length and 0.7–0.9 cm width (stem) immediately after washing and before blanching.

Blanching

In order to denature ESP and maximize sulforaphane formation, broccoli was subjected to blanching using the optimal conditions determined by Pérez et al. (2014). Broccoli florets (300 g) were immersed in 1.5 L deionized water in a thermostatic bath (Stuart, United Kingdom, Great Britain) at 57 °C for 13 min. After blanching, broccoli florets were immediately put in an ice-water bath and then crushed to obtain 0.5-cm broccoli pieces.

Experimental design

Optimization of the incubation conditions was performed through a Box–Behnken design with three central points. The experimental factors (and levels) were temperature (22 and 38 °C), incubation time (3 and 7 h) and addition of ascorbic acid (0.03 and 1 mg g^{-1} fresh broccoli). The levels in this design were chosen based on preliminary incubation experiments made to define the experimental region. The response variables were sulforaphane concentration and myrosinase activity. Table 1 shows the experimental matrix. Results are presented as average ± standard deviation.

Incubation procedure

Samples of crushed blanched broccoli were put in empty, hermetically closed flasks and were immersed in a thermostatic water bath (Trilab, Mexico City, Mexico) at the conditions stipulated by the experimental design. Two independent replicates were carried out for each condition, and each sample was analyzed in triplicate. Incubation was performed in darkness. After the treatments, the samples were kept at −20 °C until analysis.

Analytical determinations

Sulforaphane content

Sulforaphane content was assessed by reverse phase HPLC, using the method proposed by Liang et al. (2006) with some minor modifications. Samples of incubated broccoli were homogenized in a mortar, until obtaining a homogeneous meal. One gram of the sample was extracted two times with 10 mL methylene chloride (J.T. Baker, Center Valley, PA, USA), which was combined with 0.5 g anhydrous sodium sulfate (Sigma-Aldrich, Schnelldorf, Germany). The equipment was a HPLC–DAD (Shimadzu, Kyoto, Japan), and a C_{18} column (5 μm particle size, 250 × 4.6 mm) (Agilent Technologies, Santa Clara, CA, USA) was used. Quantification was carried out by comparison with a sulforaphane standard curve (0.056–6.75 μg). Organic solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany). Sulforaphane content was expressed in μmol g^{-1} (dry weight). All determinations were made in triplicate.

Myrosinase activity

Myrosinase extraction was made following the protocol described by Guo et al. (2011). Samples of incubated broccoli were homogenized in a mortar, until obtaining a

Table 1 Experimental matrix of the optimization design, sulforaphane content and myrosinase activity obtained in each run

Run	Temperature (°C)	Time (h)	Ascorbic acid (mg g^{-1})	Sulforaphane (μmol g^{-1} DW)	Myrosinase activity (U mg^{-1} protein)
R1	22	3	0.515	5.0 ± 0.5^{bcdef}	3.3 ± 0.1^{b}
R2	22	5	0.03	4.7 ± 0.1^{abcd}	3.5 ± 0.0^{b}
R3	22	5	1.00	5.2 ± 0.1^{abcde}	5.3 ± 0.0^{d}
R4	22	7	0.515	6.1 ± 0.2^{fg}	3.5 ± 0.0^{b}
R5	30	3	0.03	4.6 ± 0.7^{a}	4.9 ± 0.2^{cd}
R6	30	3	1.00	4.7 ± 0.1^{abc}	4.6 ± 0.3^{c}
R7	30	5	0.515	6.1 ± 0.3^{ef}	5.2 ± 0.1^{cd}
R8	30	5	0.515	6.4 ± 0.3^{gh}	4.7 ± 0.1^{cd}
R9	30	5	0.515	6.1 ± 0.7^{def}	5.2 ± 0.1^{cd}
R10	30	7	0.03	5.3 ± 0.2^{bcdef}	5.2 ± 0.1^{cd}
R11	30	7	1.00	5.0 ± 0.8^{cdef}	3.6 ± 0.0^{b}
R12	38	3	0.515	8.0 ± 0.3^{h}	6.1 ± 0.3^{e}
R13	38	5	0.03	7.6 ± 0.4^{h}	5.3 ± 0.3^{d}
R14	38	5	1.00	4.3 ± 0.1^{ab}	3.7 ± 0.0^{b}
R15	38	7	0.515	5.5 ± 0.7^{cdef}	2.5 ± 0.1^{a}

The superscripts letters indicate the membership to a homogeneous group, determined through the LSD multiple range test (95% confidence interval). Different letters indicate significant differences

Results correspond to the average of three replicates and the respective standard deviation

DW dry weight

homogeneous meal. After that, 0.25 g were massed and mixed with 1 mL sodium phosphate buffer (100 mM pH 6.5). The mixture was vortexed and centrifuged at 15,000×*g* during 20 min at 4 °C. The supernatant was recovered. Enzyme activity was assessed spectrophotometrically through the method described by Rakariyatham et al. (2006). One mL of substrate buffer (33 mM sodium phosphate buffer, pH 7.4 containing 10 mM sinigrin, 3 mM MgCl, 0.55 ATP, 0.72 mM NADP, 3.5 U hexokinase, and 1.75 U glucose-6-phosphate dehydrogenase) (Sigma–Aldrich, Schnelldorf, Germany) was pre-incubated for 3 min at 30 °C. Then, 100 μL of sample were added. After mixing, the reaction was monitored at 346 nm. Myrosinase activity was calculated from increasing absorbance rate, which was due to the formation of NADPH. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard. One unit of myrosinase activity was defined as the amount of enzyme that catalyzed the liberation 1 μmol of glucose per min from sinigrin under the conditions described above. Results were expressed in units per mg protein. All determinations were made in triplicate.

Statistical analyses

The significance of the statistical effects was assessed by ANOVA followed by the least significant difference (LSD) test, at 95% confidence level. The optimization design was analyzed by response surface methodology, using a second order polynomial model to describe the experimental behavior (Eq. 1).

$$\hat{Y} = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 \sum_{i<j=1}^{k} \beta_{ij} x_i x_j \quad (1)$$

In Eq. 1, $\hat{Y}$ is the predicted response; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for interception, linear, quadratic and interaction effects, respectively; k is the number of independent parameters (k = 3 in this study), and X_i, X_j are the coded levels of the experimental conditions. The model quality was assessed by the determination coefficient (R^2). The optimum incubation conditions predicted by the regression model were validated experimentally. Statistical analyses were performed with JMP 9.0.1 software (SAS Institute).

Results and discussion

The antioxidant activity of broccoli after incubation, determined through the method of Brand-Williams et al. (1995), was significantly lower ($p < 0.001$) than that of the fresh vegetable. Fresh broccoli had an anti-radical power (ARP) equal to 26.4 ± 4.4 μmol g^{-1}, while incubated broccoli had an average ARP of 21.5 ± 4.2 μmol g^{-1}. This difference may be attributed to thermal decomposition of ascorbic acid and to the loss of some antioxidant

compounds such as polyphenols through leaching in the blanching step.

The sulforaphane content and myrosinase activity obtained in each condition are given in Table 1. The superscripts letters indicate the membership to a homogeneous group, determined through the LSD multiple range test. Temperature had a significant positive effect on the response ($p < 0.05$), as well as the interaction of temperature with time ($p < 0.05$), temperature with ascorbic acid ($p < 0.05$) and the interaction of ascorbic acid with itself ($p < 0.05$). Accordingly, these factors and interactions were considered in the regression model for the optimization. The model that describes sulforaphane content as a function of the significant factors in coded levels is given in Eq. 2. Here, [S] is sulforaphane content, T is temperature, t is time and [AA] is ascorbic acid concentration.

$$[S] = 6.194 + 1.086T - 1.843Tt - 1.858T[AA] - 1.997[AA]^2 \quad (2)$$

The adjustment of the model to the experimental data gave a determination coefficient (R^2) equal to 89.88%, i.e. the model is able to explain about 90% of the data variability.

Figure 1a, b show the response surface obtained for sulforaphane concentration. Figure 1a shows that there is an optimal combination of incubation time and ascorbic acid concentration, which corresponds to 3 h of incubation and 0.22 mg ascorbic acid per g of raw broccoli. In this analysis we considered that temperature was kept at 38 °C. Figure 1b shows that low temperature and long incubation time favor sulforaphane formation. However, in the opposite directions, there was an increase of sulforaphane content. This agrees with the fact that at the low temperature level (22 °C), an increase from the low (3 h) to the high (7 h) level of immersion time results in an increase of 113% in sulforaphane content, from 3.3 to 7.0 μmol g^{-1} (dry weight). At the high level of temperature (38 °C) an increase in incubation time results in a significant decrease in sulforaphane equal to 40% [from 9.1 to 5.4 μmol g^{-1} (dry weight)]. The maximum sulforaphane content was achieved at 38 °C. According to the regression model, the optimal incubation conditions are 3 h of incubation at 38 °C in presence of 0.22 mg ascorbic acid per g fresh broccoli. In these conditions, the maximum sulforaphane content predicted by the model was 8.0 μmol g^{-1} (dry weight). This value was verified experimentally, resulting in a sulforaphane concentration equal to 8.1 ± 0.3 μmol g^{-1} (dry weight), thus agreeing with the prediction of the model. This value represents a 585% increase with respect to fresh broccoli and a 119% increase with respect to blanched broccoli, whose sulforaphane content was 1.2 ± 0.2 and 3.7 ± 0.3 μmol g^{-1} (dry weight), respectively.

Our results partially agreed with some related studies. Guo et al. (2013) reported the maximum formation of isothiocyanates by incubating homogenized broccoli sprouts at 40 °C for 3 h at pH 4.0 with the addition of 0.02 mmol EDTA and 0.16 mg ascorbic acid. Shen et al. (2010) found the maximum sulforaphane formation by incubating broccoli seeds powder mixed with distilled water and ascorbic acid at 25 °C for 8 h. The authors found

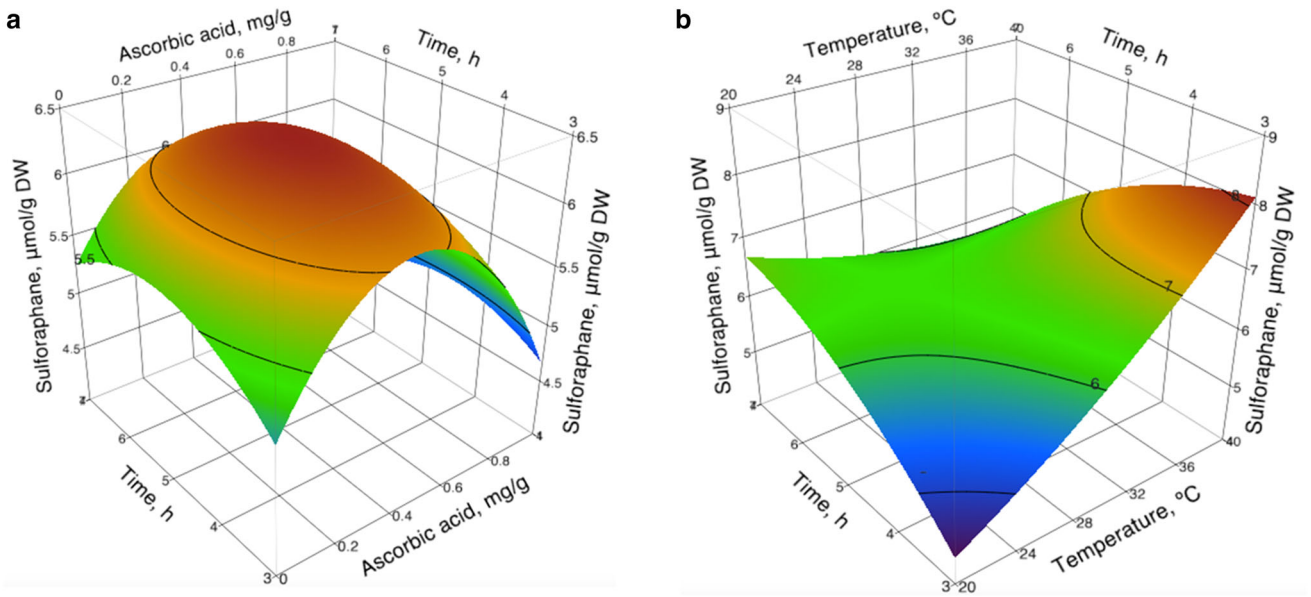

Fig. 1 Response surfaces for sulforaphane concentration obtained from the optimization design. **a** Effect of time and ascorbic acid concentration; **b** Effect of time and temperature

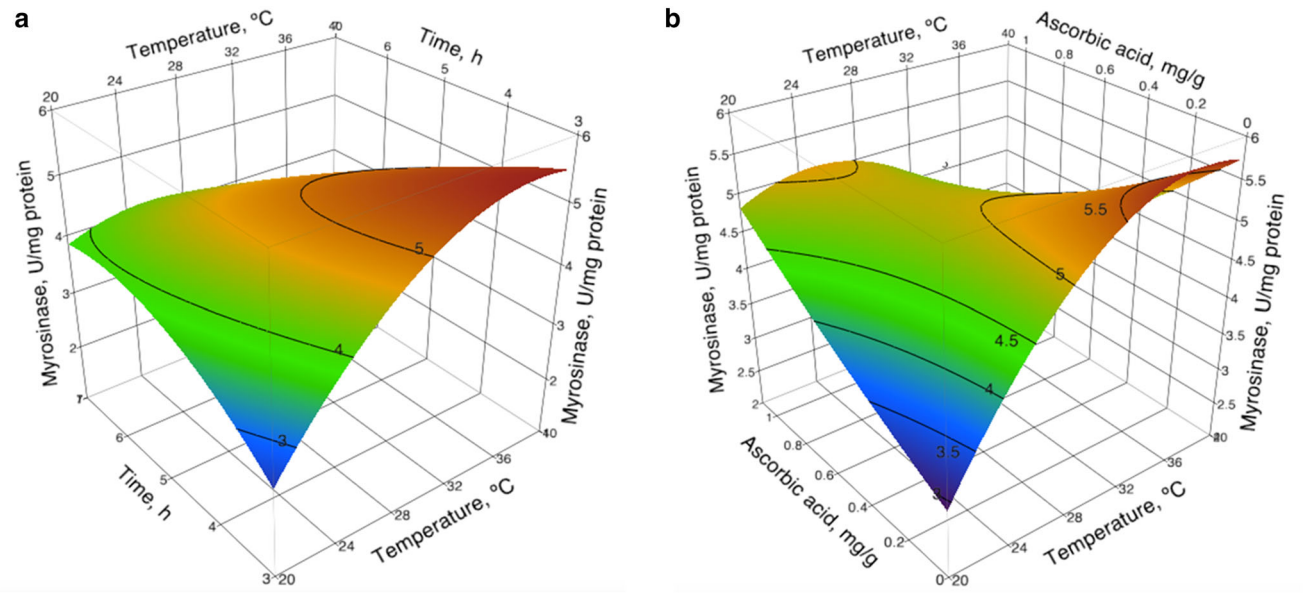

Fig. 2 Response surfaces for myrosinase activity obtained from the optimization design. **a** Effect of time and temperature; **b** Effect of ascorbic acid concentration and temperature

a glucosinolates conversion equal to 35%, while in the present work we achieved about 94% conversion of glucoraphanin, equivalent to 75% conversion of glucosinolates. The optimal ascorbic acid concentration derived from our results is higher than the reported by Shen et al. (2010), probably because that study was performed in broccoli suspensions or homogenates, where mass transfer has less hindrances.

Figure 2a, b show the response surface obtained for myrosinase activity. The high level of temperature favored enzyme activity at short incubation time and low ascorbic acid concentration. At low temperature, high concentration of ascorbic acid and long incubation times led to a higher myrosinase activity. The maximum activities were observed at the high temperature level (38 °C). The statistical analysis showed that the interaction of temperature with time and ascorbic acid had significant negative effects on myrosinase activity, with p values equal to 0.028 and 0.041, respectively. Myrosinase activity exhibited a similar behavior to that of sulforaphane concentration. Both responses were maximized at the high temperature level and short incubation time. However, despite myrosinase showed the highest activity in these conditions, it is not possible to ensure that these are the optimal conditions with certainty, since the best incubation conditions are too close to the limits of the experimental region. Nevertheless, as shown in Fig. 2a, the optimal incubation conditions should be close to 38 °C and 3 h incubation. These conditions agree with the conditions that maximize sulforaphane content.

Conclusion

The optimal incubation conditions predicted by the regression model were temperature equal to 38 °C during 3 h in the presence of 0.22 mg ascorbic acid per g fresh broccoli. The predicted maximum sulforaphane concentration (8.0 μmol g^{-1}) was confirmed experimentally yielding a value of 8.1 ± 0.3 μmol g^{-1} (dry weight). This represents an increase of 585% with respect to fresh broccoli and of 119% with respect to blanched broccoli, equivalent to a conversion of 94% of glucoraphanin. This is, to our knowledge, the first study about the effects of the incubation conditions of broccoli on sulforaphane formation. These findings could be used as basis for designing a process to significantly increase sulforaphane content avoiding artificial chemical synthesis. The compound could probably be isolated from broccoli in the future, and be delivered as a nutraceutical or functional ingredient in further elaborated foods.

Acknowledgements This work was funded by Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt) Grant No. 1130384. Carmen Pérez is grateful to Comisión Nacional de Investigación Científica y Tecnológica (Conicyt Grant for Ph.D. studies, Chile).

References

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

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT: Food Sci Technol 28:25–30

Elbarbry F, Elrody N (2011) Potential health benefits of sulforaphane: a review of the experimental, clinical and epidemiological evidences and underlying mechanisms. J Med Plants Res 5:473–484

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

Guo R, Yuan G, Wang Q (2011) Effect of sucrose and mannitol on the accumulation of health-promoting compounds and the activity of metabolic enzymes in broccoli sprouts. Sci Hortic 128:159–165. doi:10.1016/j.scienta.2011.01.014

Guo Q, Guo L, Wang Z, Zhuang Y, Gu Z (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

Jones RB, Frisina CL, Winkler S, Imsic M, Tomkins RB (2010) Cooking method significantly effects glucosinolate content and sulforaphane production in broccoli florets. Food Chem 123:237–242. doi:10.1016/j.foodchem.2010.04.016

Liang H, Yuan QP, Dong HR, Liu YM (2006) Determination of sulforaphane in broccoli and cabbage by high-performance liquid chromatography. J Food Compos Anal 19:473–476. doi:10.1016/j.jfca.2005.11.005

Matusheski NV, Juvik JA, Jeffery EH (2004) Heating decreases epithiospecifier protein activity and increases sulforaphane formation in broccoli. Phytochemistry 65:1273–1281. doi:10.1016/j.phytochem.2004.04.013

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

Rakariyatham N, Butr-Indr B, Niamsup H, Shank L (2006) Improvement of myrosinase activity of *Aspergillus* sp. NR4617 by chemical mutagenesis. Electron J Biotechnol 9:379–385. doi:10.2225/vol9-issue4-fulltext-13

Shapiro TA, Fahey JW, Wade KL, Stephenson KK, Talalay P (2001) Chemoprotective glucosinolates and isothiocyanates of broccoli sprouts: metabolism and excretion in humans. Cancer Epidemiol Biomark Prev 10:501–508

Shen L, Su G, Wang X, Du Q, Wang K (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

Van Eylen D, Bellostas N, Strobel BW, Oey I, Hendrickx M, Van Loey A et al (2009) Influence of pressure/temperature treatments on glucosinolate conversion in broccoli (*Brassica oleraceae* L. cv *italica*) heads. Food Chem 112:646–653. doi:10.1016/j.foodchem.2008.06.025

Wang GC, Farnham M, Jeffery EH (2012) Impact of thermal processing on sulforaphane yield from broccoli (*Brassica oleracea* L. ssp. *italica*). J Agric Food Chem 60:6743–6748. doi:10.1021/jf2050284

Zhou Q, Chen B, Wang X, Wu L, Yang Y, Cheng X, Hu Z, Cai X, Yang J, Sun X, Lu W, Yan H, Chen J, Ye J, Shen J, Cao P (2016) Sulforaphane protects against rotenone-induced neurotoxicity in vivo: involvement of the mTOR, Nrf2, and autophagy pathways. Sci Rep 6:32206. doi:10.1038/srep32206