Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 43 (2006) 19–22
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Effects of metal ions on myrosinase activity and the formation of sulforaphane in broccoli seed

Hao Liang, Qipeng Yuan*, Qian Xiao

Department of Pharmaceutical Engineering, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China

Available online 24 April 2006

Abstract

Effects of six metal ions on the formation of sulforaphane and the liberation of glucose upon hydrolysis of glucoraphanin by myrosinase at neutral pH were studied. The yields of sulforaphane and glucose were determined by HPLC. Copper ion and magnesium ion decreased the yields of sulforaphane and glucose. Ferrous ion and ferric ion inhibited the formation of sulforaphane, but had no effects on the liberation of glucose. Calcium ion increased the yield of glucose liberation, but inhibited the formation of sulforaphane. Only zinc ion was beneficial to the liberation of glucose and accelerated the formation of sulforaphane at initial reaction intervals.

Keywords: Myrosinase; Sulforaphane; Metal ions; Broccoli seed

1. Introduction

Glucosinolates are sulfur-containing glycosides detected in varying amount in cruciferous vegetables that have been tested so far. When cruciferous vegetables are ground or chopped, myrosinase enzyme (thioglucoside glucohydrolase, EC3.2.3.1) and glucosinolates come into contact. Myrosinase breaks the β-thioglucoside bond of glucosinolate molecules, producing glucose, sulfate, and a diverse group of aglycone products. The resultant aglycones then undergo non-enzymatic, intramolecular (Lossen) rearrangement to yield isothiocyanates, thiocyanates or nitriles (Fig. 1).

Glucoraphanin (4-methylsulfinybutyl glucosinolate), a glucosinolate found in broccoli (*Brassica oleracea* L. var.), produces mostly sulforaphane (4-methylsulfinybutyl isothiocyanate) and nitrile when it is hydrolyzed by myrosinase. Significantly, sulforaphane can reduce the incidence of a number of forms of tumor and induce cell cycle arrest and apoptosis in various experimental models [1–4]. In contrast, nitrile has not shown any health benefit and may be toxic to normal cells [5]. Because of the substantial difference of sulforaphane and nitrile, preferential conversion of glucoraphanin to sulforaphane is necessary to maximize the health benefit from eating broccoli and the yield of sulforaphane for the future production. However, an optimum hydrolyzed condition for the formation of sulforaphane has not been reported yet.

It is generally accepted that low pH, ferrous ion and epithiospecifier protein (ESP) are beneficial to induce the formation of nitrile when glucosinolates are broken by myrosinase [6,7]. The pH during hydrolysis has been found to influence both the activity of myrosinase and the ratio of isothiocyanates to nitriles formed in the disrupted tissue of cruciferous vegetables. Ferrous ion, also favorable to form nitrile, has no inhibitory effect on the liberation of glucose from sinigrin by myrosinase in an acidic condition. It has been reported that copper ion and ferric ion have effects on the degradation of glucosinolates by myrosinase [8,9]. However, it is not clear that these two ions affect the activity of myrosinase or the arrangement of aglycones. Myrosinase from *Sinapis alba* was found to be a zinc-containing enzyme [10]. Thus, it is possible that zinc or other metal ions may also exist in myrosinase from broccoli and play a very important role in the hydrolysis of glucosinolates. In order to obtain a better understanding of the role of metal ions in enzyme catalysis and aglycone rearrangement of glucoraphanin, six metal ions were selected to investigate the relation of metal ions to the formation of sulforaphane.

* Corresponding author. Tel.: +86 10 6443 7610; fax: +86 10 6443 7610.
E-mail address: yuanqp@mail.buct.edu.cn (Q. Yuan).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.03.008

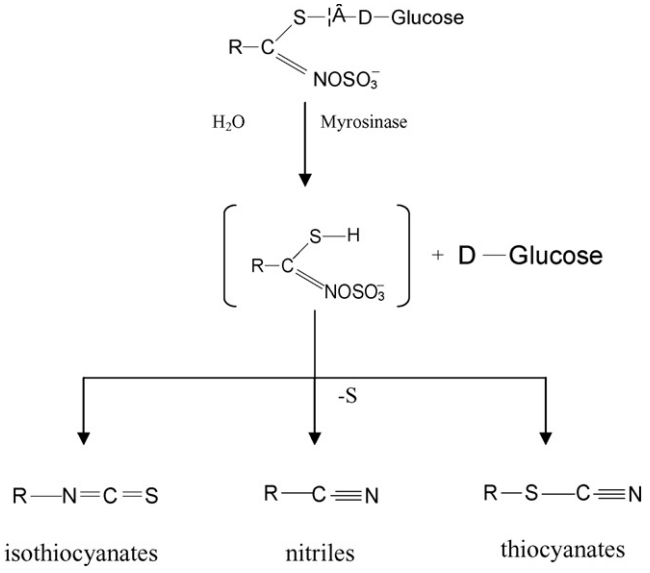

Fig. 1. Enzymatic conversion of a glucosinolate to an aglucone intermediate by myrosinase, and subsequent conversion to an isothiocyanate, nitrile, or thiocyanate.

2. Materials and methods

2.1. Materials

Zhongqing II broccoli (*B. oleracea* L. var.) seeds were kindly provided by the Vegetables and Flowers Institute of the China Academy of Agriculture Science. Sulforaphane standard was purchased from Sigma Chemical Co. (St. Louis, MO.). Acetonitrile was HPLC grade. Methylene chloride, hexane, Tris, HCl, EDTA, metal chloride and anhydrous sodium sulfate were reagent grade.

2.2. Preparation of sulforaphane extract

Seeds of the broccoli cultivar 'Zhongqing II' were ground into powder. Ground seed was defatted three times with excess hexane and dried in a fume hood. Following this, defatted seed (0.5 g) was hydrolyzed by adding water in a ratio of 5:1 water/defatted seed (w/w), and the mixture was allowed to autolyse at room temperature. After autolysing, this mixture was extracted two times with 10 ml methylene chloride, which was combined and salted with 0.5 g anhydrous sodium sulfate. The methylene chloride fraction was dried at 30 °C under vacuum on a rotary evaporator. The residue was dissolved in 5 ml acetonitrile and was then filtered through a 0.22 μm membrane filter prior to injection into HPLC.

2.3. Determining sulforaphane content by reverse phase HPLC

Sulforaphane was analyzed with a Hitachi HPLC apparatus equipped with Hitachi model L-7100 pumps, L-7420 tunable absorbance detector, reversed phase C_{18} column (250 mm × 4.6 mm, 5 μm, Diamodsil™). The solvent system consisted of 20% acetonitrile in water for 5 min, then changed linearly over 20 min to 100% acetonitrile, and maintained 100% acetonitrile for 2 min to purge the column. Column oven temperature was set at 30 °C. The flow rate was 1 ml/min, and 10 μl portions were injected into the column. Sulforaphane was detected by absorbance at 254 nm.

2.4. Analyzing the content of the producing glucose

Defatted seed (0.5 g) was hydrolyzed by adding 2.5 ml water at room temperature. The mixture was centrifuged at 8000 rpm for 5 min, and the supernatant was filtered through a 0.22 μm membrane filter. Ten microlitres of this supernatant was injected into HPLC. Glucose was detected by refractive index using a Hitachi differential refractometer. The column was NH_2 column (4.6 mm × 150 mm, 5 μm, Inertsil™), and column oven temperature was maintained 35 °C. The mobile phase was 75% acetonitrile in water, and the flow rate was 1 ml/min.

3. Results and discussion

3.1. Effect of EDTA on the formation of sulforaphane

For these experiments, the broccoli seed was employed as the substrate, because the glucoraphanin level in most broccoli seed represents up to more than 90% of the glucosinolates [11]. Fig. 2 shows a typical HPLC chromatogram of volatile cleavage products of the glucosinolate mixture.

It is possible that myrosinase from broccoli contains some metal ion which is beneficial to maintain the enzyme structure or enhance the activity of myrosinase. EDTA solution was added into the defatted seed powder in order to chelated metal ions in broccoli seed cell when it autolysed. EDTA was dissolved with Tris–HCl buffer (pH 7.2, 25 °C) for the sake of stabilizing pH of reaction system. It is very interesting that the yield of sulforaphane was almost invariable and only slightly decreased with increasing the concentration of EDTA (Fig. 3). It means that myrosinase in broccoli seed may not contain any metal ions which significantly influence the final yield of sulforaphane.

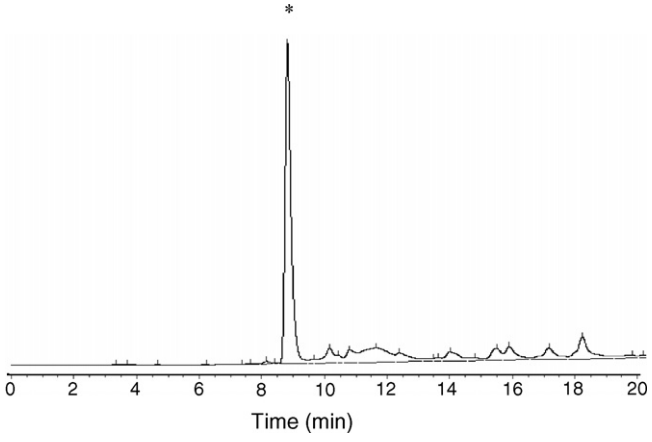

Fig. 2. HPLC chromatogram of volatile cleavage products of the broccoli seed glucosinolates. Note: (*) the peak of sulforaphane.

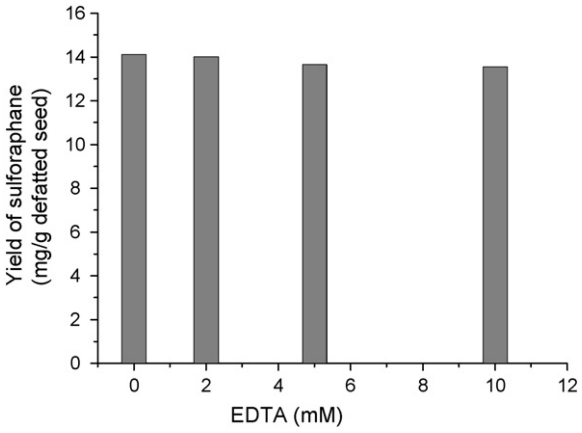

Fig. 3. Yield of sulforaphane at different concentrations of EDTA.

3.2. Effects of metal ions on the formation of sulforaphane and the liberation of glucose

Effects of six metal ions on the formation of sulforaphane and the liberation of glucose were shown in Fig. 4. Metal ions may affect the aglycone rearrangement or the activity of myrosinase enzyme. Analyzing the contents of sulforaphane and glucose in the hydrolyzed products is helpful to understand the hydrolysis mechanism of glucoraphanin with metal ions. In order to eliminate the influence of pH and anion, all selected metal ions were chloride and were dissolved with Tris-HCl buffer (pH 7.2, 25 °C).

Effects of these six metal ions on the formation of sulforaphane and the liberation of glucose can be divided into four groups: (1) Zinc ion was beneficial to the formation of sulforaphane and the liberation of glucose, although the increase of glucose was higher than that of sulforaphane. It indicated that zinc ion can significantly enhance the activity of myrosinase and thus it may be a cofactor of myrosinase. (2) Both magnesium ion and copper ion decreased the yield of sulforaphane and glucose. There was a maximum inhibition on the formation of sulforaphane at 2 mM magnesium ion. Increasing the concentration of magnesium ion, the yield of sulforaphane increased slightly. But the liberation of glucose decreased linearly with the increasing concentration of magnesium ion. It is possible that the aglycone was prone to form sulforaphane and

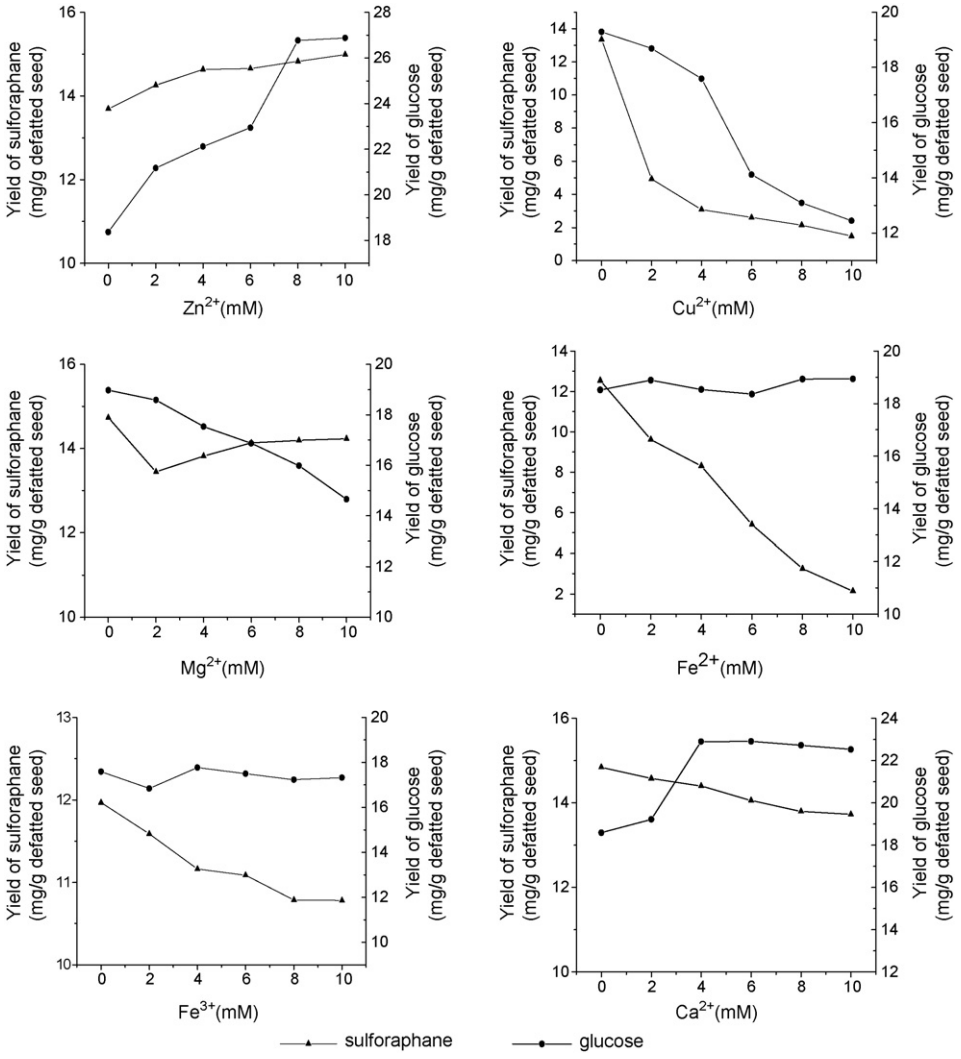

Fig. 4. Effects of six metal ions on the formation of sulforaphane and the liberation of glucose.

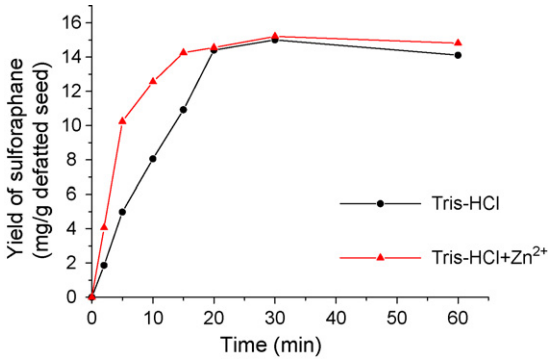

Fig. 5. The formation of sulforaphane in the presence or absence of zinc ion at different intervals.

the myrosinase activity was inhibited at high concentration of magnesium ion. However, the inhibition of copper ion was significantly greater than that of magnesium ion. Copper ion can inhibit both the formation of sulforaphane and the deliberation of glucose. At low copper ion concentration (2 mM), the yield of sulforaphane decreased significantly, but the yield of glucose decreased slightly. The yield of sulforaphane and glucose were both diminished vastly at high concentration (10 mM). It indicated that copper ion not only influence the aglycone rearrangement but also inhibit the enzyme activity. (3) Ferrous ion and ferric ion only inhibited the formation of sulforaphane, but had no effect on the liberation of glucose from glucoraphanin by myrosinase. Uda et al. [6] observed that effect of ferrous ion was notably depressed at pH 6.5 and disappeared at pH 7.5. In our study, it was found that ferrous ion can also significantly inhibit the formation of sulforaphane at pH 7.2, and the inhibition of ferrous ion was strengthened with the increase of ferrous ion concentration. Moreover, the inhibition of ferrous ion to the formation of sulforaphane was higher than that of ferric ion. (4) Effect of calcium ion was very interesting, because of the increase of glucose liberation and the decrease of sulforaphane formation. When calcium ion existed, the aglycone of glucoraphanin possibly tended to form other byproducts. The role of metal ions in the formation of aglycone products is not fully understood, but interference with the Lossen rearrangement caused by affinity of metal ions for the sulfide group and nitride group of the primary product is one possible explanation.

3.3. Effects of zinc ion on the formation of sulforaphane at different reaction intervals

In the above experiment, glucoraphanin in broccoli seed meal was allowed to hydrolyze for a relatively long time (2 h). It is possible that metal ions only accelerate the hydrolyzed reaction, but have slight effect on the balance of the reaction system. In the second experiment, hydrolyzing of glucoraphanin in broccoli seed was going on at different times and in the same concentration of zinc ion solution. Fig. 5 shows the formation of sulforaphane from glucoraphanin in broccoli seed powder in the presence or absence of 2 mM zinc ion at different intervals. In the absence of zinc ion, the formation of sulforaphane increased linearly with the time of the hydrolysis of broccoli seed powder until 20 min. With zinc ion existing, the formation rate of sulforaphane increased significantly till 20 min, but the final yield of sulforaphane slightly increased as compared to the one without zinc ion. The same behavior observed at two situations was that the concentration of sulforaphane decreased slightly after 30 min. The same phenomenon was found by Uda et al. [6]. Sulforaphane probably reacted to give another product, but none was detected in our work.

4. Conclusions

In this paper, six metal ions (Zn^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}) were selected to investigate the effects of metal ions on the enzymatic system converting the aglycone of glucoraphanin to sulforaphane in a neutral pH condition. Copper ion and magnesium ion decreased the yield of sulforaphane and glucose. Ferrous ion and ferric ion vastly inhibited the formation of sulforaphane, and were not concerned in the liberation of glucose. Calcium ion increased the liberation of glucose, but inhibited the formation of sulforaphane. Furthermore, Cu^{2+}, $Fe^{2+} > Fe^{3+} > Ca^{2+} > Mg^{2+}$ inhibited the formation of sulforaphane distinctly. Only zinc ion was beneficial to the formation of sulforaphane and the liberation of glucose, and accelerated the formation of sulforaphane at initial reaction. It is assumed that zinc ion may improve the activity of myrosinase or favor the aglycone of glucoraphanin converting to sulforaphane. More research will be required to elucidate the precise mechanism of this activation and its significance in the metabolism of glucosinolates in the intact plant.

Acknowledgement

The authors would like to acknowledge the support of Beijing Key Laboratory of Bioprocess (SYS100100421).

References

[1] Y. Zhang, T.W. Kensler, C.G. Cho, G.H. Posner, P. Talalay, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 3147.
[2] P. Talalay, Y. Zhang, Proc. Biochem. Sci. 24 (1996) 806.
[3] Y. Zhang, P. Talalay, Cancer Res. 58 (1998) 4632.
[4] L. Gamet-Payrastre, P. Li, S. Lumeau, G. Cassar, M. Dupont, S. Chevolleau, N. Gase, J. Tulliez, F. Terce, Cancer Res. 60 (2000) 1426.
[5] N.V. Matusheski, E.H. Jeffery, J. Agric. Food Chem. 49 (2001) 5743.
[6] Y. Uda, T. Kurata, N. Arakawa, Agric. Biol. Chem. 50 (1986) 2735.
[7] M. Zabala, M. Grant, A.M. Bones, R. Bennett, Y.S. Lim, R. Kissen, J. Rossiter, Phytochemistry 66 (2005) 859.
[8] C. Shin, K. Seo, K. Kang, C. Ahn, Y. Kim, K. Shim, Han'guk Sikp'um Yongyang Kwahak Hoechi. 4 (1996) 687.
[9] L.M. Searle, K. Chamberlain, D.N. Butcher, J. Sci. Food Agric. 35 (1984) 745.
[10] W.P. Burmeister, S. Cotlaz, H. Driguez, R. Iori, S. Palmieri, B. Henrisat, Structure 5 (1997) 663.
[11] M.M. Kushad, A.F. Brown, A.C. Kurilich, J.A. Juvik, B.P. Klein, M.A. Wallig, E.H. Jeffery, J. Agric. Food Chem. 47 (1999) 1541.