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ORIGINAL ARTICLE

Effect of Heavy Metal Ions and Carbohydrates on the Activity of Cauliflower (*Brassica oleracea* Var. botrytis) Myrosinase

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Myrosinase is an enzyme of cruciferous vegetables, hydrolyse glucosinolates. The breakdown products are involved in plant defence against insect and also have anti-fungal property. Myrosinase has been purified to apparent homogeneity from 5 days old germinated cauliflower seedlings having a specific activity of 12.71 units/mg proteins with 54.6 % recovery, using ammonium sulfate fractionation followed by gel filtration chromatography on Sephadex G-100. Effect of some metal ions and carbohydrates on the activity of partially purified cauliflower myrosinase was studied. Sr^{+2} at 4 mM concentration exhibited marked activating effect on the activity up to 2.7 fold while Fe^{+2} significantly inhibited. However, Sn^{+2} and Ba^{+2} increased the activity to a certain extent and then suppressed. On the other hand, some metal ions [Fe^{+2}, Fe^{+3}, Cu^{+2} and Zn^{+2}] strongly inhibited the activity even at lower concentrations. Several carbohydrates viz., glucose, fructose, sucrose, maltose and sorbitol even at comparatively higher concentrations had little detectable inhibitory effects. Activation kinetics of myrosinase in presence of Sn^{+2} and Sr^{+2} were studied between 0- 20min. The rate of reaction was almost constant till 15 min and then slight deactivation was recorded at various concentrations used.

Key words: Myrosinase, Partial purification, Metal ions, Carbohydrates, Cauliflower.

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Plant myrosinase (thioglucosidase glucohydrolase EC 3.2.3.147), is an interesting enzyme from cruciferous vegetables, catalyses hydrolysis of glucosinolate to form volatile and nonvolatile products, such as isothiocyanates, thiocyanates, oxazolindine-2-thiones, indoles and nitriles, depending on the reaction conditions and availability of the ferrous ions (Halkier and Du, 1997).

The myrosinase-glucosinolate system has

numerous functions in the plant. The glucosinolate degradation products are involved in plant defence against insects and phytopathogens, in sulfur and nitrogen metabolism and growth regulation. The breakdown products not only contribute to the aroma and flavour of crucifers, but have also been shown to display diverse and interesting biological properties such as hepatotoxic or goitrogenic, antibacterial, anti-fungal, and/or anti-carcinogenic activities (Zhang and Talalay, 1994; Lewis and Fenwick, 1987; Fahey *et al*., 1997). Myrosinase and the glucosinolates are physically separated from each other in cells, and thus hydrolysis can only occur when cells are disrupted by the processes as mastication (Verkerk *et al*., 2001; Buchwaldt *et al*., 1986 ; Lönnerdal and Janson, 1973). Myrosinase has been purified from several sources, including white mustard (*Sinapis alba*) by Bjorkman and Janson (1972), cress (*Lepidium sativum*) by Durham and Poulton (1989), yellow mustard (*Brassica juncea*) by Ohtsuru and Hata, (1972) and rape seed (*Brassica napus*) by Lönnerdal and Janson (1973). The role of myrosinase mediated breakdown products of glucosinolates in reducing various types of cancers in human and animal cell models have been widely studied by Conaway and associates (2002) & Chiao and associates (2002). Clinical studies have also shown that the bioavailability and absorption of isothiocyanates from raw *brassica* vegetables is much greater than that from cooked ones in which myrosinase have been inactivated (Rouzaud *et al*., 2003). Significantly, sulforaphane (a type of isothiocynate) can reduce the incidence of a number of forms of tumour and induce cell cycle arrest and apoptosis in various experimental models (Zhang *et al*., 1994; Talalay and Zhang, 1996; Zhang and Talalay, 1998; Gamet-Payrastre *et al*., 2000). In addition, some metal ions have been shown to regulate the formation of sulphoraphane from glucosinolates in broccoli (Liang *et al.,* 2006). With these in view, present study was undertaken to explore the possible direct effects of some heavy metal ions and carbohydrates on the activity of partially purified myrosinase from cauliflower seedlings.

MATERIALS AND METHODS

Chemicals and materials

Sinigrin monohydrate and BSA were purchased from Sigma- Aldrich Corporation, USA. Sephadex G-100 was from Pharmacia Fine Chemicals, Uppsala, Sweden. All other metal chloride and carbohydrates were of Analytical Grade either from HiMedia or Sisco Research Laboratories, India. All solutions were prepared using quartz double distilled water. Seed of brassica vegetables was obtained from Seed Production Division, Indian Institute of Vegetable Research (IIVR) Varanasi, India.

Purification of myrosinase

Sample Preparation

The seeds (5 gm.) were surface-sterilized by rinsing in 70% ethanol and after that the seeds were kept for 5-10 minutes in 1.0% sodium hypochlorite followed by exhaustive rinsing with sterile distilled water. These seeds were plated on moist and sterile paper towels in petri plates at a density of about 10 seeds/cm^2 and allowed to grow at 25°C under white fluorescent light on a 16:8 h light/dark cycle. After 5 days, sprouts were gently collected from the surface of the paper towels and immediately homogenized in 25 ml of potassium phosphate buffer (20mM, pH 7.4). The crude extract thus obtained was filtered through properly washed and pre-chilled muslin cloth and was centrifuged at 15000g, for 15 min at 0-4°C to remove the insoluble

materials.

Enzyme and protein assay

Myrosinase activity was determined by measuring the rate of decrease in absorbance at 227 nm resulting from the hydrolysis of sinigrin as per method described by Palmieri and associates (1982). Protein concentrations were determined by the method of Lowry and associates (1951) with BSA as a standard. Specific activities are expressed as units/mg of protein.

Ammonium Sulphate fractionation

The crude myrosinase was precipitated with fine crushed solid ammonium sulphate. The supernatant was saturated up to 60% with $(NH_4)_2SO_4$ under continuous stirring and maintaining the pH 7.4 by adding 0.1M KOH drop by drop at 4°C. The suspension was centrifuged at 15000 g for 15 min. at 0-4°C. The pellet thus obtained was dissolved in extraction buffer (20mM, pH 7.4). The final volume was made up to 10.5 ml. This fraction was extensively dialyzed using thoroughly washed and pre activated dialysis tube with continuous stirring for 24 hours at 0-4°C against the same buffer.

Gel filtration chromatography

The dialyzed extract was further purified using gel filtration chromatography (Sephadex G-100). The Sephadex beads were activated using extraction buffer for 72 hours with washing from time to time. Activated beads were poured slowly in a glass chromatography column with the help of a glass rod to avoid trapping of air bubbles. The sample obtained from previous step was applied to the pre-equilibrated column. Eluents were collected in different fraction tubes of 1ml. The fractions containing myrosinase activity were pooled and concentrated against solid sucrose. The concentrated sample was diluted with extraction buffer according to need. The above sample was stored in a refrigerator at 0-4°C and used throughout this study.

Analytical electrophoresis

Slab gel electrophoresis was carried out at 25°C in a mini protein electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). 8% Native PAGE was carried out according to the method of Laemmli (1970). The bands were visualised by Coomasie Brilliant blue R-250 and in order to check the purity of the enzyme preparation, activity staining was done by the method of Lönnerdal and Janson (1973).

Effect of metal ions and carbohydrates on myrosinase activity

A stock solution of each of the desired reagent was prepared in double distilled water. The residual myrosinase activity of suitably diluted enzyme was determined in the presence of varying concentration of these reagents added in the standard assay mixture.

RESULTS AND DISCUSSION

Partial purification of enzymes:

Among the three steps selected for the study, protein precipitated in the range of 0-60% saturation of ammonium sulphate (having a specifiic activity of 2.3 U/mg of protein was dialysed and selected for further seperation on sephadex G-100 column. Taking in to account the myrosinase obtained in a single symmetrical peak from fraction 9-15, 10.08 fold purification was achieved with recovery of 54.6 % yield (Table-1 and Fig. 1). The purification results reported here are comparable with those of Xian & Kushad (2005) who purified myrosinase from horse radish roots by ammonium sulphate precipitation to approximately

7.3 fold (specific activity 26.0 units/mg protein) with 87% yield.

Effect of metal ions on cauliflower myrosinase activity:

Various metal ions such as Fe^{+3}, Zn^{+2}, Sn^{+2}, Sr^{+2}, Ba^{+2}, Cu^{+2} and Fe^{+2} at various concentration ranging from (1 to 40mM) were tested for activation/ inhibition effects. Sn^{+2}, Sr^{+2} and Ba^{+2} had strong activation effects on myrosinase activity (Fig. 2). Among these three metal chlorides Sr^{+2} had a strongest activation effect and activity continuously increases from 0-4mM and maximum activity was recorded at 4mM (nearly 2.7 times of the control) afterward activity decreases slowly and the enzyme became completely inactive at 40mM . Tani and associates (1974) however, reported that Sr^{+2} ions were an inhibitor of bacterial myrosinase. This difference in the behaviour might be due to the structural difference in the myrosinase. Plant myrosinase had a rather large molecular weight as compared to the bacterial enzyme. Similarly, stannous ions had maximum activity (1.67 times of control) at 2mM concentration and beyond which there was a decrease in the activity and no activity was recorded at 6mM onwards. Similar observations of minor activation were also reported in bacterial myrosinase by Tani and associates (1974). Ba^{+2} showed a little activation of enzyme and maximum activation was recorded at 10mM.

A concentration dependent and time dependent inhibition in the enzyme activity was observed in case of Fe^{+3}, Fe^{+2}, Zn^{+2} and Cu^{+2} (observed from 1-40mM, Fig. 3). Fe^{+2} made a complete loss in the activity at 1mM while Fe^{+3} showed 50% residual activity as that of control at 1 mM and there was zero activity at 4 mM. Fig.3 indicated that ferrous and ferric ions were strong inhibitor of cauliflower myrosinase. In earlier study Jwanny and associates (1995) reported the similar finding with Fe^{+2} at 1mM concentration. A similar trend with respect to concentration dependence was observed in case of Cu^{2+} where more than 2/3rd activity was lost at 1mM and only 8% of maximum activity (maximum in control) was remained at 2mM and no activity was recorded at 4mM. Among Fe^{+3}, Fe^{+2}, Zn^{+2} and Cu^{+2}, Zinc ion had a mild inhibitory effect on myrosinase and had complete loss in activity at 6mM (Data not shown). The enzyme forms a dimer stabilized by a Zn^{2+} ion and is heavily glycosylated by Burmeister and associates (1997). Similar finding was also reported by Jwanny and associates (1995) and Tani and associates (1974). The inhibitory effect may be due to the blocking of the binding site of the substrate or instability of enzyme- substrate complex.

Liang and associates (2006) also reported inhibitory effect of ferrous and ferric ion on Myrosinase activity. Moreover, the inhibition by ferrous ion in the formation of sulforaphane was higher than that of ferric ion. Similar finding was also recorded with Cu^{2+}, copper ion not only influenced the aglycone rearrangement but also inhibited the enzyme activity.

Effect of carbohydrates on myrosinase:

The effect of various carbohydrates such as glucose, fructose sucrose, maltose and sorbitol was studied in the concentrations range of 5 to 50mM (Fig. 4). The concentration of these sugars exhibiting detectable inhibition in cauliflower myrosinase was found to be 20mM suggesting that all these carbohydrates were weak inhibitor of Myrosinase. Amongst these carbohydrates, glucose was a strongest inhibitor and > 50% activity was lost at 50mM concentration while sucrose had a weakest inhibition. The trend of inhibition was recorded as glucose > fructose > sorbitol > maltose > sucrose at

50mM (Data not shown). Tani and associates (1974) also reported the inhibitory effect of various carbohydrates at 100mM. Shikita and associates (1999) reported that glucose was a weak inhibitor of myrosinase; it may be due to the binding of carbohydrates to the products.

Time –course of myrosinase action in presence of Sn^{+2}:

As has been described earlier in Fig.2, Sr^{+2} exhibited activation of myrosinase activity in the concentration range of 1-6 mM. Considering this, a time–course of effect of metal ion at the concentration of 1 & 2 mM was studied. The metal ion was added in the reaction mixture and the progress of reaction was studied for 20 min (Fig. 5). The result revealed that rate of reaction was persistent till 13 min and after which little decrease in enzyme activity was noted till 20 min in both the concentrations selected.

Time-course of effect of Sr^{+2} on the activity of the myrosinase:

As described earlier (Fig. 2), Sr^{2+} in the concentration range of 1-20 mM had activation effect on myrosinase, and the activation was significant with 4 mM Sr^{2+}. In order to explore the time-course of activation, three concentrations viz., 1,2 and 4mM, were selected and studied for 20 min. Fig. 6 indicated that at 4mM concentration of Sr^{2+}, the rate of reaction was better than lower concentrations. Time dependent rate of reaction was recorded at these concentrations. Nearly till 15 min the rate of reaction was constant and further, little decrease in activity was detected (remains 80% of maximum) till 20 min.

The results reported above are suggestive of the fact that heavy metal ions at suitable concentrations are probably stabilizing the enzyme such that the myrosinase continues to act on its substrate for longer duration of time, without significant decrease in the activity.

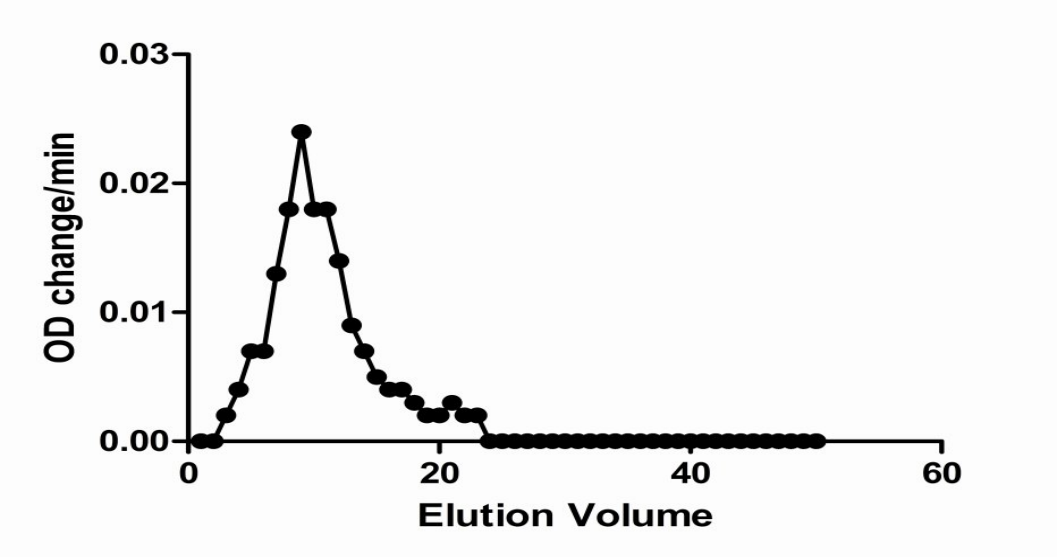

Figure 1: Purification profile in cauliflower myrosinase in Sephadex G-100 gel filtration chromatography.

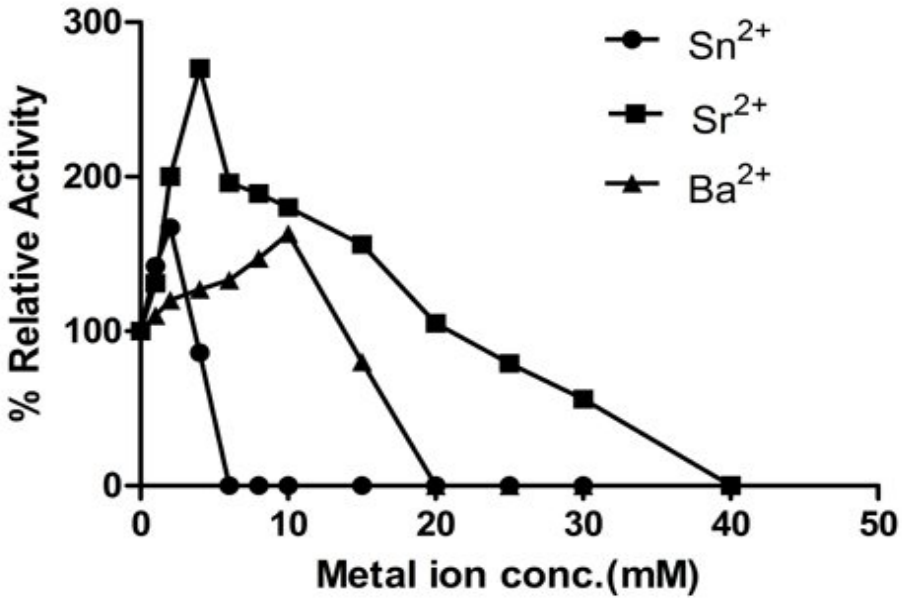

Figure 2: Effects of metal ions on the activity of myrosinase of cauliflower seedling.

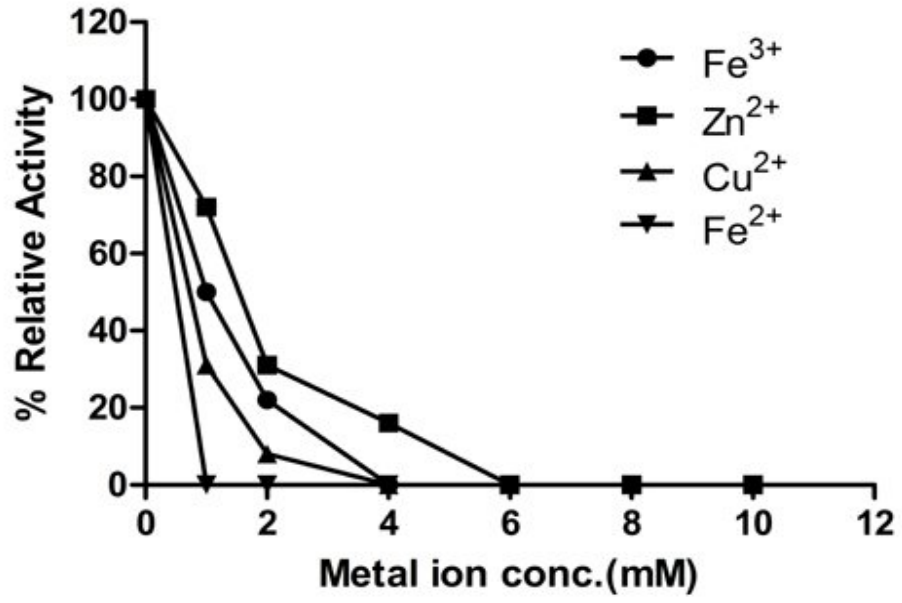

Figure 3: Effects of metal ions on the activity of myrosinase of cauliflower seedling.

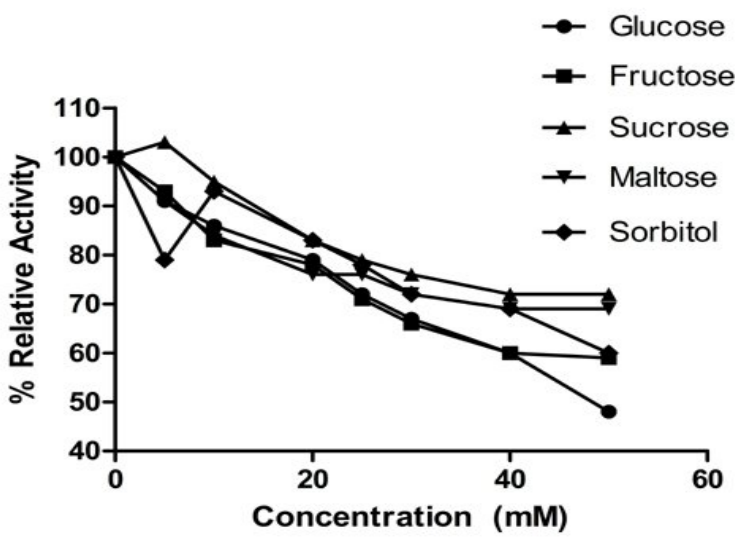

Figure 4: Effects of carbohydrates on the activity of myrosinase of cauliflower seedling.

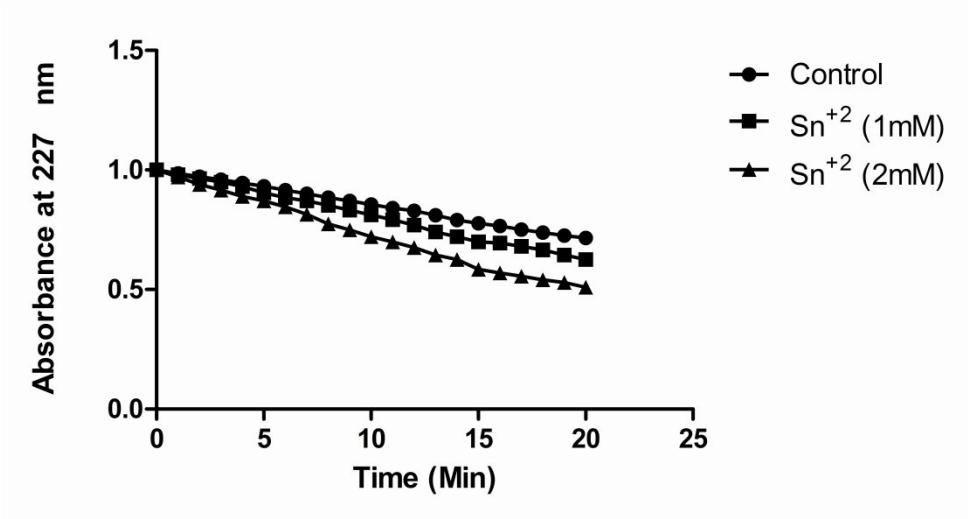

Figure 5: Rate of reaction of myrosinase at different concentration of Sn^{+2} with control at different time interval.

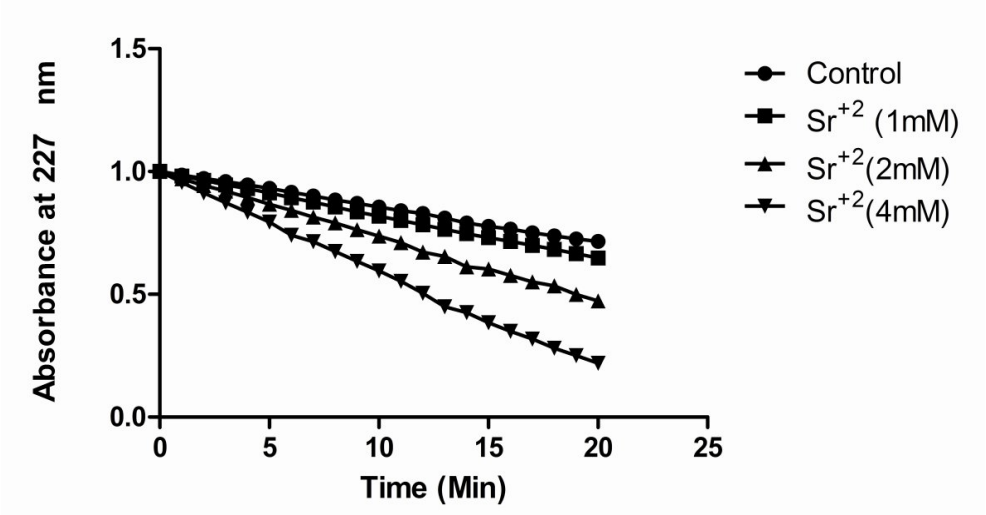

Figure 6: Rate of reaction of myrosinase at different concentration of Sr^{+2} with control at different time interval.

Table 1. Purification of myrosinase from Cauliflower Seedling.

Sample	Volume (ml)	Total activity (unit)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude	22.5	12.09	9.54	1.26	-	-
0-60% Amm. Sulphate	10.5	8.31	3.612	2.3	1.65	62.6
Dialysis	9.0	8.22	3.50	2.34	1.66	62.0
Sephadex G-100	3.0	7.25	0.57	12.71	10.08	54.6
CD(5%)		0.597	0.203	0.133	0.115	1.32

CONCLUSION

The cauliflower myrosinase was partially purified by ammonium sulphate fractionation followed by gel filtration chromatography to a purification factor of 10.08 with 54.6% yield. In this paper, several metal ions viz., Fe^{+3}, Zn^{+2}, Sn^{+2}, Sr^{+2}, Ba^{+2}, Cu^{+2} and Fe^{+2} & few carbohydrates i.e. glucose, fructose, sucrose, maltose and sorbitol were selected to investigate the effects on the enzymatic activity. Further research will be required to elucidate the precise mechanism of this activation and its significance in the metabolism of glucosinolates in the intact plant.

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