EFFECTS OF SULPHATE AND MICRONUTRIENTS ON THE MYROSINASE ENZYME SYSTEM. I. ENZYME ACTIVITIES AND ACTIVATION BY ASCORBIC ACID.

T. GAUTEPLASS, S. VISVALINGAM AND A.M. BONES*

UNIGEN Center for Molecular Biology, Department of Botany, University of Trondheim, MTFS, N-7005 Trondheim, Norway, FAX: 47-73-598705, E.mail: Atle.Bones@Unigen.Unit.No

ABSTRACT

The myrosinase (thioglucoside glucohydrolase) enzyme system in species of Brassicaeae is coded by genes belonging to at least two subgene families of thioglucosidases. The expression of myrosinases varies and is dependent on developmental stage, organ examined and environmental factors.
The effect of sulphate and the micronutrients iron, zinc, copper and manganese on the myrosinase activities and activation by ascorbic acid were determined in plants seeded and cultured in vitro on defined media. Eleven media compositions were tested at 4 developmental stages and in 4 different organs of the plants examined. Expression of myrosinases was in general high in plants cultured on a medium supplemented with sulphate. Culture on a medium without sulphate containing salts gave a marked decline in myrosinase activity in 10 day or older plants. Of the other nutrients tested, ferrous ion was the one that influenced the myrosinase activity most. Of the organs examined bud was the most sensitive and showed the highest variation in myrosinase activity. Highest enzyme activities were found when plants were cultured on medium containing both sulphate and ferrous ions.

INTRODUCTION

The myrosinase-glucosinolate system consists of more than 100 substrates (glucosinolates) and a group of degrading enzymes, the myrosinases (thioglucoside glucohydrolase, EC 3.2.3.1) and cofactors. The chemistry of glucosinolates has been studied in detail. Information about myrosinases is, however, still limited. Myrosinase has been purified to homogeneity from seeds of *Brassica napus* L. and *Sinapis alba* L. Based on electron microscopic studies, immunocytochemical and immunogold-EM studies, evidence for the cellular and subcellular localisation of myrosinase has been given (Thangstad *et al.* 1990; Bones *et al.* 1991). Myrosinase is localised to protein bodies/vacuoles of idioblasts named myrosin cells (Thangstad *et al.* 1991). The occurrence and distribution of myrosinase activity at different developmental stages and in different organs of species of Brassicaceae has been examined by Bones (1990). Myrosinases are coded by a gene family with subfamilies, and cDNA clones (Xue *et al.* 1992, Chadchawan *et al.* 1993) and genomic genes (Thangstad *et al.* 1993) belonging to two subgroups (*Myr1* and *Myr2*) have been characterized. The myrosinase-glucosinolate system is thought to have an important role in the plants metabolism and protection system against pathogens and pests (Chew 1988; Schnug 1990). Recently, Bones *et al.* (1994) reported that sulphate can induce differential expression of myrosinases.

In this paper we report that the myrosinase activity in *Sinapis alba* L plants depend on the nutritional status of the plants. To be able to exclude other variable environmental parameters plants were cultured in vitro under controlled growth room conditions.

EXPERIMENTAL

Plant material.

Seeds of *Sinapis alba* cv. Trico were kindly provided by Svalöf Weibull AB, Svalöf, Sweden. Seeds were sterilised and seeded in glass pots on complete MS-medium (Murashige and Skoog 1962) or on modified MS-media. The modified media include all MS-components with exception of the element specified, E.g. $FeSO_4$ - medium contain all MS-salts except ferrous sulphate. TS-medium was made by replacment of all sulphate containing salts with the same amount of chloride containing salts. S^--medium is lacking all sulphate containing salts of the original MS-medium. All media were solidified with 0.5% (w/v) agar and

supplemented with 30 g l^{-1} sucrose . *In vitro* cultured *Sinapis alba* L. plants were harvested 5, 10, 15 and 21 days after seeding. Plant parts were separeted and proteins extracted under strictly similar conditions.

Myrosinase and protein assay.

Myrosinase was measured as liberated glucose with the GOD-Perid assay (Boehringer) and/or the hexokinase assay (Sigma). Activation of myrosinases by ascorbic acid was measured with the hexokinase assay in the presence of 0.4 mM ascorbic acid. Protein was measured by the Bio-Rad (California, USA) reagent using bovine serum albumin as standard.

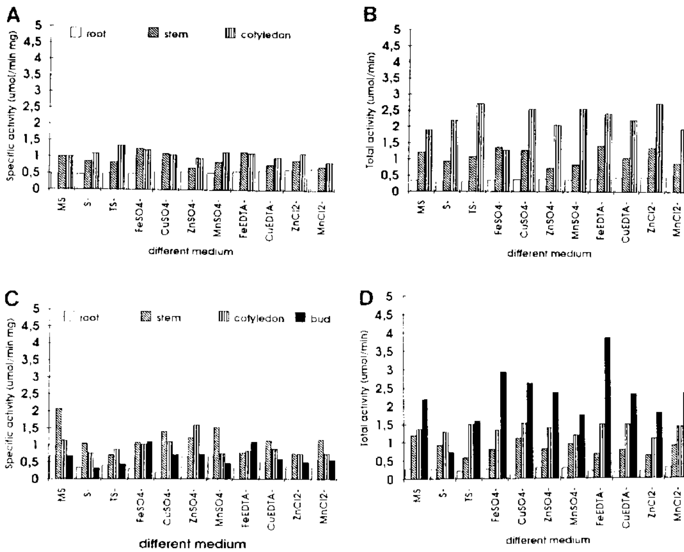

FIG. 1. Specific (**A** and **C**) and total (**B** and **D**) myrosinase activity with extracts from **A-B**: 5 and **C-D**: 15 day old *Sinapis alba* plants cultured *in vitro* on medium as indicated. Notice the organ and time specific expression of the myrosinases detected. Plant organ is indicated. Total activity is expressed as µmol min^{-1} and specific activity as µmol min^{-1} mg^{-1}. Values represent average of duplicates.

Expression of myrosinases activity

The total activity of myrosinase, measured as liberation of glucose, was different when seedlings were cultured on medium which lack specific elements (Fig. 1). With the exception of sulphate, very little is known about effects of nutrients on the expression of myrosinases. Schnug (1990) reported that a decreasing sulphur supply of *Brassica napus* plants resulted in a decrease in free sulphate and glucosinolate concentrations and an increase in myrosinase activity. Such an increase in myrosinase activity during sulphur stress could have the function of a remobilization of sulphate sulphur from glucosinolates, because sulphate and isothiocyanates can be utilized as sulphur sources in the primary metabolism of the plants. In our experiments with 5 to 21 day old plants of *Sinapis alba*, sulphur stress gave in general a decline in the myrosinase activity (Fig.1). Similar results was obtained for sulphate in an earlier study (Bones *et al.* 1994). Of the ions examined sulphate and ferrous ions influenced myrosinase activity most. Sulphate starvation gave a decline and ferrous ions starvation an increase in enzyme activity. It should be stressed that the effects are dependent both on developmental stage and organ examined (Fig.1).

Activation by ascorbic acid

Effects of ascorbic acid on the myrosinase activity in the samples was investigated by the hexokinase assay and the bariumsulfate assay (see e.g. Bones *et al.* 1994). The level of activation of myrosinases was dependent on the organ examined and also varied at different developmental stages. Highest activation of myrosinases could be observed in roots and buds (Fig. 2). The activation by ascorbic acid was highest in the 21 day old plants (data not shown).

Conclusion

Our results show that the expression of myrosinases in roots, leaves, cotyledons and buds is dependent on the nutrients available for the plants. Of the elements studied ferrous ions and sulphate had most influence on the myrosinase activity. The highest total myrosinase activity was found in buds of 15 day old plants (Fig.1). With the exception of sulphate, plants cultured under nutritional stress in general show an increase in total myrosinase activities.

Multiple forms of myrosinase in some species of Brassicaceae have been reported earlier. Our examination of the enzyme activation by ascorbic acid do support that different organs of the plant contains different myrosinase species or combinations of enzymes. Furthermore, the expression of these are organ and time specific (see e.g. Bones *et al.* 1994). As we have shown in the present paper, the myrosinases in buds and roots are more activated by ascorbic acid than the myrosinases in cotyledon. The myrosinase-glucosinolate system is thought to be a part of the plants protection system against pathogens. If a high expression of myrosinases are favourable for an optimal protection against pathogen attacks, cultivation on a optimal fertilized soil may facilitate an increased protection.

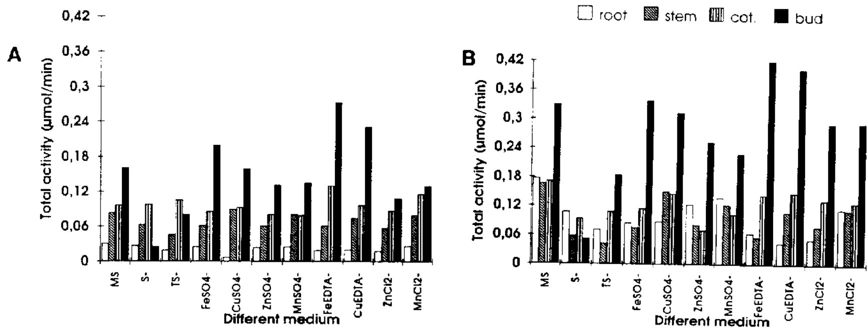

FIG. 2. Total myrosinase activity in extracts from 15 day old plants measured with the hexokinase assay. **A** without ascorbic acid and **B** with 0.4 mM ascorbic acid in the assay solution. Notice the organ dependent activation.

ACKNOWLEDGEMENTS

Financial support from The Norwegian Research Council (NFR) and Hydro AS are gratefully acknowledged.

REFERENCES

Bones, A.M. (1990). Distribution of β-thioglucosidase activity in intact plants, cell and tissue cultures and regenerated plants of *Brassica napus* L. *J. Exp. Bot.* **41**, 737-744

Bones, A.M., Thangstad, O.P., Haugen, O.A., Espevik, T. (1991). Fate of myrosin cells: Characterization of monoclonal antibodies against myrosinase. *J. Exp. Bot.* **42**, 1541-1549

Bones, A.M., Visvalingam, S., Thangstad, O.P. (1994). Sulphate can induce differential expression of thioglucoside glucohydrolases (myrosinases). *Planta* **193**, 558-566.

Chadchawan, S., Bishop, J., Thangstad, O.P., Bones, A.M., Mitchell-Olds, T. and Bradley, D. (1993). *Arabidopsis* cDNA sequence encoding myrosinase. *Plant Physiol.* **103**, 671-672

Chew, F.S. (1988). Biological effects of glucosinolates. In: *Biologically active natural products for potential use in agriculture*, pp. 155-181, Cutler, H.G. ed. Am. Chem. Soc., Washington

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497

Schnug, E. (1990). Glucosinolates - fundamental, environmental and agricultural aspects. In: *Sulphur nutrition and sulphur assimilation in higher plants*, pp. 97-106, Rennenberg, H., Brunold, C., de Kok, L.J., Stulen, I. eds.. SPB Academic Publishing, Hague

Thangstad, O.P., Evjen, K., Bones, A.M. (1991). Immunogold-EM localization of myrosinase in Brassicaceae. *Protoplasma* **161**, 85-93

Thangstad, O.P., Iversen, T.H., Slupphaug, G., Bones, A.M. (1990). Immunocytochemical localization of myrosinase in *Brassica napus* L. *Planta* **180**, 245-248

Thangstad, O.P., Winge, P., Husebye, H., Bones, A.M. (1993). The myrosinase (thioglucoside glucohydrolase) gene family in Brassicaceae. *Plant Mol. Biol.* **23**, 511-524.

Xue, J., Lenman, M, Falk, A., Rask, L. (1992). The glucosinolate-degrading enzyme myrosinase in Brassicaceae is encoded by a gene family. *Plant Mol. Biol.* **18**, 387-398