QUANTITATIVE ANALYSIS OF GLUCOSINOLATES AFTER THEIR ENZYMATIC DESULFATATION ON ION EXCHANGE COLUMNS

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Today gas chromatography (GC) of trimethylsilyl derivatives of glucosinolates (GSLs) belongs to the prefered methods which are used for the analysis of GSLs in rapeseeds. Unfortunately the sulfate rest which is split off from the GSL molecule during the derivatization process may interfere with the following GC-separation especially if the defatted seed or leaf meal contains less than 10 µmol/g GSLs (Thies, unpublished). Following our search for an enzyme which hydrolyses the sulfate ester bond without attack on the S-glycosidic bond of the GSLs, we found such a catalyst in the digestive tract of the edible snail Helix Pomatia. Besides crude enzyme solutions which may be prepared according to Fabergé (1945), commercially available products which are offered as β-glucoronidases and arylsulfatases from H. Pomatia are suitable sources for the "GSL-sulfatase" activity. Throughout all experiments described below the sulfatase type H-1, Sigma S 9626 was used.

Properties of the Helix sulfatase

After the action of the enzyme on sinigrin in water followed by addition of Ba-acetate, a white precipitate of Ba-sulfate is formed. This reaction is suitable for the detection of the sulfatase on electropherograms (Gmelin, 1978; private comm.). The hydrolysis of sinigrin and other GSLs may also be traced by thin layer chromatography with butanol/i-propanol/acetic acid/water (3:1:1:1) as developing solvent and 2 % thymol in ethanol/conc. sulfuric acid (9:1) as stain reagent. The travelling velocities of desulfo-GSLs in this system have nearly the twofold values than those of the parent GSLs. From eleven GSLs, which have been tested as substrates of the sulfatase until now, all were hydrolysed. Among them were alkyl-, alkenyl-, OH-alkenyl-, methylthio-, methylsulfinyl-, methylsulfonyl-, aryl- and indolyl-derivates. As expected the gaschromatographic retention times of silylated desulfo-GSLs had the same values as silylated GSLs. Attempts to hydrolyse the S-glycosidic bond of desulfated GSLs by a myrosinase from Sinapis alba were unsuccessful. This result is in agreement with observations of Ettlinger and Dateo (1961).

With sinigrin in ethylenediamine-acetate buffer as substrate the sulfatase had its highest activity at pH 5.8. The optimum substrate concentration was 0.15 mmol/l (Fig. 1). The initial reaction velocity as well as the yield of desulfo-sinigrin liberated from the parent sinigrin depended on the enzyme concentrations with optima at 0.01 % and 0.005 % resp. (Fig. 2). The enzyme activity is strongly inhibited by sulfate-, phosphate-, copper-, lead- and silver-ions. No activating compounds could be detected until now. Solutions of the sulfatase (0.05 % in water with 0.002 % chlorohexidine diacetate as antisepticum), stored during six weeks at room temperature, showed no detectable loss of activity despite of the formation of voluminous precipitates during this time. The activity was measured using arbitrary units (velocity of decrease of the extinction at 227.5 nm after adding 200 µl of the enzyme solution to 2 ml 0.15 mmol/l sinigrin in 33 mmol/l ethylenediamine-acetate buffer pH 5.8 (compare Fig. 3 and text below Fig. 2).

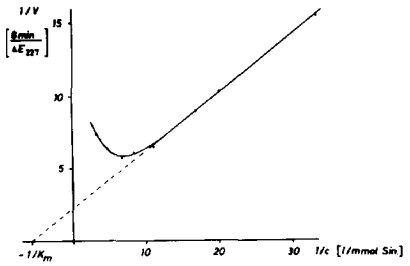

FIG. 1

Lineweaver-Burk-diagram of the sulfatase preparation "Sigma type H-1" with sinigrin as substrate. To 1.9 ml 33 mmol/l ethylenediamine-acetate buffer pH 5.8 50 µl 0.2 % enzyme in water and 50 µl sinigrin solution of different concentrations were added.

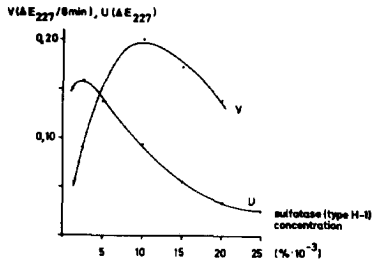

FIG. 2

Dependence of the initial reaction velocity V and the final turnover U of sinigrin to desulfo-sinigrin upon the concentration of the "Sigma H-1 sulfatase". To 1.95 ml of 0.15 mmol/l sinigrin in 33 mmol/l ethylenediamineacetate buffer pH 5.8 50 µl enzyme solutions of different concentrations were added. The arbitrary unit ΔE (227.5 nm) = E (sinigrin) - E (sinigrin + desulfo-sinigrin) had to be chosen because the accurate extinction coeff. of desulfo-sinigrin at 227.5 nm at present time is not known (see also text below Fig. 3)

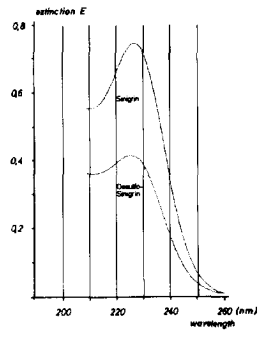

FIG. 3

Extinction curves of sinigrin and desulfo-sinigrin (0.1 mmol/l each in water, 1 cm pathlength) between 210 and 260 nm wave-length, measured against water. From the picture an extinction coefficient of about 3900 l/mol·cm for desulfo-sinigrin can be estimated.

DESULFATATION OF GSLS ON ANION EXCHANGE COLUMNS

The hydrolysis of the sulfate ester bond of GSLs is also "on column" possible. This technique has the advantage that the neutral desulfo-GSLs which can be eluted from the exchanger by water are free from other substances except a small amount of the enzyme. Replacing the steps 15 to 23 of a previously published method (Thies, 1977) the following procedure may be recommended. To 2 ml water extract from defatted seed or leaf meal containing about 1 µmol GSLs 100 µl Pb-acetate and Ba-acetate (each 0.5 mol/l water) are added. After mixing and centrifugation 500 µl of the supernant are passed through 20 mg (dry weight) Sephadex DEAE A-25 in a Pasteur pipet. Rinsing the column with 1 ml 0.02 mol/l pyridineacetate not only removes the neutral compounds but also changes the distribution of the GSLs on the column which results in a 20 % higher yield of desulfo--GSLs. After the addition of 50 µl 0.05 % "H-1 sulfatase" in water and in-

cubation over night at room temperature the desulfo-GSLs are eluted with 0.5 ml water. 200 µl of 6 mol/l imidazoleformiate followed by 1 ml water are sufficient for the regeneration of the column.

ANALYTICAL ASPECTS

Because of the high purity of the desulfo-GSLs isolated according to the above described method, a comparison of the absorbancies of the column effluents at 227.5 nm may be used for estimation of their total contents of desulfo-GSLs (Fig. 4 and 5). The formation of trimethylsilyl derivatives of desulfo-GSLs for GC analyses proceeds markedly faster than the silylation of GSLs: 1.5 hours at room temperature or 15 min at 80°C are sufficient.

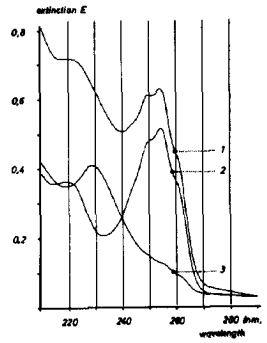

FIG. 4

Extinction curves of desulfo-GSLs in water isolated from water extracts of rapeseed meals after enzymatic desulfatation of the parent GSLs "on column". (1) and (2): Desulfo-GSLs derived from meals containing 100 µmol/g and 10 µmol/g GSLs. 50 µl of the column effluents (=10 % of the total effluent volume) were mixed with 2 ml water and measured against water. (3): Extinction curve of sample 1 measured against sample 2 (difference spectrum).

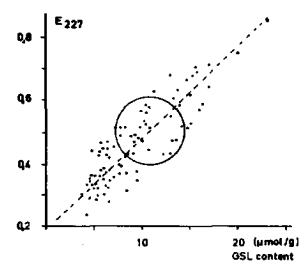

FIG. 5

Dependence of the extinctions at 227.5 nm of desulfo-GSLs derived from different seed sources according to the "on column-desulfatation technique" upon their GSL-content determined by gas chromatography. Determination of the E-values: 50 µl of the effluent volumes were mixed with water and measured against a sample prepared under same conditions from a standard meal (variety "Erglu"). Before taking of the readings the zero point of the recorder was set to 50 % of the chart width (E at 227.5 nm = 0.5; air to air measurement).

DISCUSSION

The history of the "sinigrin sulfohydrolase" is as confusing as our present knowledge of the different arylsulfatases (Dodgson and Rose, 1975). Apart from earlier and later discussions concerning the nature of the myrosinase it seems to be likely that Ishimoto and Yamashina (1949) and Takahashi (1960) were the first working with a "true" sinigrin sulfohydrolase (from the marine mollusc Charonia Lampas). But because of some uncertainties and lack of further research the enzyme sinigrin-sulfohydrolase was deleted from the list of enzymes in the 3rd report of the Enzyme Commission in 1972. Now it has to be shown whether the GSL-sulfohydrolase activity in H. Pomatia is a property of one of the known arylsulfatases or of a new enzyme. Our efforts to separate the sinigrin sulfohydrolase

activity from the phenolphthaleindisulfate hydrolase activity by different chromatographic techniques failed until now. But this may be due to experimental inadequaties, it is remarkable that we were not able to detect sinigrin hydrolase activities in three - compared to H. Pomatia-phylogenetic - elder molluscs and in Aerobacter Aerogenes all which are known as sources of arylsulfatases.

ACKNOWLEDGEMENT

The author is indepted to Prof. R. Gmelin, Berlin, for many fruitful discussions and generous gifts of several glucosinolates and desulfo-sinigrin.

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