STUDIES ABOUT PEROXIDASE PATTERNS IN HIGH- AND LOW-GLUCOSINOLATE RAPESEED

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INTRODUCTION

Enzymes involved in the transfer reactions of oxygen seem to have some importance for the formation of glucosinolates. Aminoacids with modified sidechains are the precursors of this products. In the case of progoitrin - the predominant glucosinolate in 0-rapeseed - 2-amino-6-(methylthio)caproic acid-is incorporated into the molecule (Lee and Serif 1970). At the stage of oxydation to the hydroxyaminoacid may be a metabolic block in the biosynthesis of glucosinolates in low-glucosinolate varieties. In precursor feeding experiments it could be shown, that the next intermediate product on the pathway to progoitrin - 5 methylthiopentanal oxime-is effectively incorporated into progoitrin by plants and parts of plants of the 00-variety Bronowsky (Josefsson 1973). In studies about the biosynthesis of cyanogenic glucosides, which is very similar to the glucosinolate synthesis, the oxydation step from the aminoacid to the hydroxyaminoacid could be assigned to a membrane bound enzyme (Halkier et al. 1989). It is also likely, that the biosynthesis of glucosinolates takes place in vegetative parts of the plant, but not in the seeds and that the glucosinolates have to be transported into the embryo by active transportmechanisms, which are also a possible block for the enrichment of glucosinolates in the seed (Gijzen et al. 1988). So a peroxidase like enzyme, that seems to be bound on particles and that occurrs as well in vegetative parts of the plant as in seeds might be an important factor in the biosynthesis of glucosinolates and as a tool for the distinction of high- and low-glucosinolate varieties. An enzyme with peroxidase activity, that was only present in the electrophoretic patterns of the seed extracts of 00-varieties, could be found. Some of its biochemical properties allowing an estimation of its importance for the biosynthesis, especially its reactions with solid particles, are subject of this presentation.

MATERIAL AND METHODS

Seeds and seedlings from the low-glucosinolate varieties Annika, Arabella, Bronowski, Ceres, Cobra, Glumander and a further variety and from the high-glucosinolate-varieties Belinda, Bienvenu, Elvira, Gundula, Jet Neuf, Mirander and Primander were used for the investigations.

Preparation of seed extracts: In 0,4 ml of buffer(0,012 M tris-glycin; 0,5 or 0,05 M tris citrat) 0,1 g of seed grains is homogenized. For extraction at lower pH-values in the range from 7,5 to 5,0 the buffer solutions are acidified by addition

of citric acid. Extracts are cleared by centrifugation and filtration about cellulose acetate filters (0,45 µm). Acidification of extracts is done by addition of citric acid, elevation of pH by a concentrated tris solution (1 g/2 ml).

Preparation of single corn extracts: Each corn is homogenized in 30 µl of 0,012 M tris-glycine-buffer (pH = 8,3) on a spot-plate. After 2 h the turbid liquid is sucked up by a pipette and used for electrophoresis. For testing the influence of ascorbic acid the grains are homogenized in degassed buffer, casually the joined extracts were filtered under N_2-atmosphere through cellulose acetate filters (0,45 µm).

Preparation of seedling extracts: Approximately 30 seedgrains per sample are submerged by tap water for 2 h and distributed on wet filterpaper. After 192 h of germination and growth, the whoole seedlings are harvested and homogenized in a glasstube. The extracts are filtered through cellulose acetate filters (0,45 µm) and diluted 1:1 by volume with 1 M tris-citrate-buffer (pH = 8,0). In some cases unfiltered extracts diluted with 1 M tris-citrate-buffer (pH = 8,0 or 5,0) are used for electrophoresis.

Disc-electrophoresis: Separating-gel: 10%-polyacrylamide gel crosslinked with 0,8 g BIS/1 in 0,375 M tris-citrate-buffer (pH = 8,3). Adjustment to lower pH is done by addition of citric acid. Stacking-gel: 5%-polyacrylamide gel crosslinked with 3 g BIS/1 in 0,12 tris-citrate-buffer (pH = 6,6). Electrode-buffer: 0,012 M tris-glycine-buffer (pH = 8,3).

Gradient-gel-electrophoresis: Linear gradient, 5 - 20% polyacrylamide in 0,375 M tris-citrate-buffer (pH = 8,3). Stacking gel and electrode buffer as in discelectrophoresis.

Visualization of peroxidase like enzymes: Enzymes are visualized by ortho-dianisidine in 2 M Na-acetate-acetic acid buffer (pH = 5,0) and 10 µl H_2O_2 (30%) per 100 ml). For determination of the pH-Optimum of peroxydases the pH in the dying solution is adjusted by Na-hydroxyde or acetic acid.

RESULTS

Seed extracts: From unfiltered samples in 0,012 M tris-glycine buffer (pH = 8,3) an electrophoretic pattern with two bands of peroxidase activity are obtained. In the following the faster running zone will be called A and the slower one B. In high-glucosinolate rape seed the weak band B disappeares completely if filtered samples are subjected to electrophoretic investigation (Fig. 1). Addition of mercaptoethanol or SDS to the unfiltered sample does not enhance the activity of this band. In contrary a decrease with higher concentrations of this reagents is absorved. Also an extended extraction time and intensified homogenization of the grains in buffer solution leads to complete disappearance of zone B. By extraction with 0,5 M tris-citric-acid buffer with different pH-values it may be seen, that the enzyme is visible in samples with pH greater or equal 6,5. Below this pH it disappears in samples of low- as well as of high-glucosinolate varieties. If samples are extracted with 0,05 M buffer, the pH-influence of the crushed grains is predominant, so that in 0-varieties the zone disappears in nearly all cases, while in 00-varieties it is present. If the acid is added to the filtered sample the band is visible down to a pH of 5,0. The

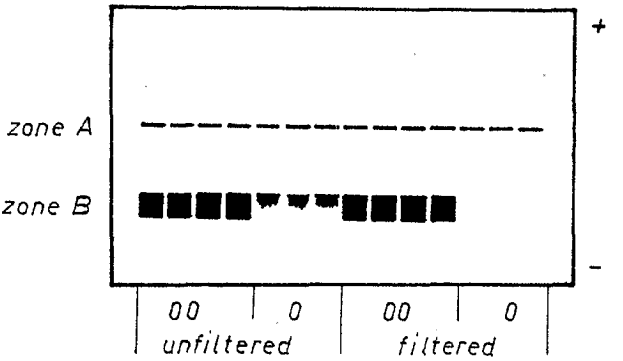

Fig. 1. Disappearance of zone B in elektrophoretic patterns of 0-varieties

enzyme is inactivated irreversibly in the presence of solid particles during extraction below pH = 6,5. Adjustment of pH ≥ 8,0 in samples extracted in buffer of pH 5,0 to 6,5 does not reactivate the enzyme in the electrophoretic patterns. Ascorbic acid does not affect the enzyme if added in a concentration of 1 mg/ml extract to a sample extracted in a 0,5 M buffer above pH = 6,5.

Single corn extracts: Peroxidase active zones A and B are found. Especially band B is always present. Differences between varieties are observed mainly in the frequency of occurance of band A. The results of the calculation of the genetic distance according to the formula of Hedrick does not show any correlation to the glucosinolate content of the varieties. The disappearance of band B is induced by addition of ascorbic acid in a quantity of 1 mg/ml extract (5 mM) selectively in samples of high glucosinolate varieties. In filtered samples the enzyme is not affected by ascorbic acid. If solids, filtered out from low-glucosinolate varieties, are resuspended in the clear extract of high glucosinolate varieties or 00-varieties with relatively high glucosinolate content, zone B disappeared when ascorbic acid was added to the suspension before electrophoresis.

Seedling extracts: The two most intensive peroxidase active bands of seedlings have the same Rf-values as the zones A and B of the seeds in the patterns of discelectrophoresis. They are also equal in molecular weight as it could be determinated by gradient gel electrophoresis. The faster running zone of the high-glucosinolate varieties is followed by a tail of peroxidase activity characteristic for 0-varieties. The electrophoretic behaviour of the slower running zone is influenced by SDS in the extraction buffer. The enzyme in the seed extract in the contrary showed no reaction on the addition of SDS. The difference between the slower moving peroxidase bands in seeds and seedlings is also evident in the following facts. The zone of seedling extracts remains equaly unchanged when the pH in the turbid or clear extract is lowered, or ascorbic acid is added. Also the pH-optimum for the seed enzyme is estimated in the range of 5,0 and for the peroxidase of the seedlings in the range of 6,0.

DISCUSSION

The most notheworthy result is the disappearance of the zone B from seed extracts selectively in high glucosinolate varieties. Though the mechanism of this inactivation is not established sc far, some experimental details may characterize the reaction and help to estimate the importance of this peroxidase like enzyme in the biosynthesis of glucosinolates. Of special interest is the necessity of suspended solids in some of the experiments, that suggests, that there might be a binding of the enzyme to membranes located mainly in the crude particles of the homogenizate. In the beginning of this work, two possibilities were taken into consideration: hydrophobic binding and disulfide linkages caused by oxidation. To the first point there is no reference, because no activation by SDS was detected although there are some references dealing with activation of peroxidases in plant and animal tissue by SDS (Pick et al 1987; Moore and Flurkey 1990). The second one seemed to be more probable, because the intensified homogenization in a mortar accompanied with intensive contact to air-oxygen, led to the total loss of the peroxidase active band B in high glucosinolate varieties. Further the addition of ascorbic acid to homogenizates in presence of oxygen caused the disappearance of zone B in 0-varieties. These facts supported the assumption of the occurrence of an enzyme which is bound by oxydizing influences to membranes of high-glucosinolate varieties. So far our experimental results do not support evidence, that the enzyme may be involved in the membrane-bound biosynthesis of glucosinolates. Binding of the enzyme to solids by disulfide linkages is not demonstrable, because addition of mercaptoethanol did not reactivate the enzyme.

In the beginning of the work an influence of low pH seemed not to be very probable, because the enzyme has a pH-optimum of 5,0. Extraction with buffers of different pH on the contrary showed clearly that the enzyme was bound by a pH-induced irreversible reaction with the solids.

CONCLUSIONS

Ascorbic acid in the presence of oxygen did not catalyze the oxidation of the thiol groups to disulfide linkages but it activated the desintegration of glucosinolate (Mac Leod and Rossiter 1987). This reaction is accompanied by acidification setting free potassium hydrogensulfate. The disappearance of zone B in high-glucosinolate varieties is caused by the decline to a lower pH in consequence of the higher amount of glucosinolate desintegrated followed by the exhaustion of the buffer. The enzyme occurs only in the seeds and not in the vegetative parts of the plant. Therefore a participation in the biosynthesis of glucosinolate can be excluded.

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