Distribution of Myrosinase in Rapeseed Tissues

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ABSTRACT

Immunoctochemical studies on Brassica napus (rapeseed) tissues using a monoclonal antibody against myrosinase (thioglucoside glucohydrolase) showed that the enzyme was only present in a small number of cells. In the developing embryo, scattered myrosinase-containing cells were present in both cotyledons and axis. The enzyme accumulated in these cells during the later stages of seed development, approximately from day 20 until day 40 after pollination. Parallel staining with the immunocytochemical technique and a histochemical method identified these cells as myrosin cells. Myrosinase appeared to be located outside the myrosin grains, although the occasional association with the membrane of the grains also was noted. In leaves, petals, and siliques, scattered parenchyma cells were stained in the mesophyll as well as in the vascular tissue. In young leaves, guard cells also contained myrosinase. The enzyme was also present in xylem cells of the stem.

The glucosinolates are a group of about 100 naturally occurring low mol wt compounds found mainly in members of the Cruciferae. They consist of a thioglucoside linked to a variety of side chains which are usually amino acid derivatives. Coexisting with the glucosinolates in the plant tissue is the enzyme myrosin or thioglucoside glucohydrolase (EC 3.2.1.1). This enzyme catalyzes the hydrolysis of glucosinolates to yield glucose, sulfate and, depending on the glucosinate species, any one of the following products: isothiocyanates, thiocyanates, nitriles, cyanopropithiokanes, or oxazolidine-2-thiones (2, 8, 14). Myrosinase from rapeseed has been purified and characterized. It consists of several isoforms. Various degrees of glycosylation account for at least part of the charge heterogeneity seen in this enzyme (10). By the use of monoclonal and polyclonal antibodies, two main types of myrosinase were found in rapeseed protein extract: 75 kD subunits occurred as free dimers, whereas 70 and 65 kD myrosinases existed in complexes with two 52 and 50 kD myrosinase-binding proteins (9). The function of the latter proteins is not known.

Hitherto there has not been a method for direct identification of myrosinase in tissue sections. On the basis of morphology and by certain staining techniques like Millon's reagent and Saffranin-fast green, myrosin cells or protein-accumulating idioblasts have been identified (4, 5, 15). In these cells, certain globular structures, myrosin grains, were stained (13, 18) and the "myrosin" was assumed to be myrosinase (19). To localize the enzyme in situ by electron microscopy, some investigators have taken advantage of the possibility of forming an electron-dense precipitate of barium- or lead-sulfate during the hydrolysis of glucosinolates at the sites of myrosinase activity. The distribution of the precipitate thus formed was then assumed to represent the location of the myrosinase (6). The enzyme was thereby detected in the majority of the cells in the embryo where it appeared to be localized to the plasma membrane (12).

Later observations using subcellular fractionation suggested that myrosinase is present in the cytoplasm but has a remarkable tendency to adhere to membrane surfaces (11), while the glucosinolates are found inside the myrosin grains/vacuoles. This distribution of myrosinase as a cytosolic enzyme agrees well with the hypothesis of a different subcellular localization of the enzyme and of the glucosinolates.

In the present study, we have used antibodies made against myrosinase to detect the enzyme in the embryo during seed development and germination, as well as in adult plants of Brassica napus.

MATERIALS AND METHODS

Plant Material

A dihaploid line of rapeseed (Brassica napus), Svalöfs Karat (Svalöf AB, Svalöv, Sweden), was used in this study. Seeds were germinated at 25°C in the dark for 2 to 8 d in a constant-environment cabinet. Plants were grown in soil in a greenhouse with 18 h light and 6 h darkness.

Monoclonal and Polyclonal Antimyrosinase Antibodies

Myrosinase was purified from rapeseed by a combination of ammonium sulfate precipitation and ion exchange chromatography. The resulting enzyme preparation consisted of a dimer of 75 kD chains. This material was used as antigen for the production of monoclonal and polyclonal antibodies. The monoclonal antibodies were selected for their ability to precipitate myrosinase activity or to react with myrosinase in Western analysis. One of these monoclonal antibodies, 3D7, as well as a rabbit polyclonal antiserum, K505, reacted with myrosinase species with subunits of molecular masses 75, 70, and 65 kD. Quantitative immunoprecipitations with K505 showed that this antiserum precipitated at least 80% of the total myrosinase activity in a rapeseed protein extract. 3D7 and K505 were selected for use in immunocytochemical studies because they reacted well with rapeseed tissues after

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Figure 1. The presence of myrosinase in cells of rapeseed embryos 35 to 40 d after pollination, as detected by immunohistochemical staining. In (a) a rabbit antiserum (K505), in (b) the preimmune rabbit serum, in (c) a monoclonal antibody (3D7) against myrosinase, and in (d) a monoclonal antibody against human β2-microglobulin (as a control) were used. Scale bar equals 10 μm.

Immunocytochemistry

Whole seeds or tissues cut into small pieces (1 × 2 mm) were fixed in 10% (w/v) formaldehyde in PBS for 2 h to 7 d at 4°C. Samples were then dehydrated through a graded series of alcohol followed by incubation in xylene before infiltration and embedding in histowax (tissue embedding medium, pellets m.p. 56–58°C, Histolab, Gothenburg, Sweden). Sections (4 μm thick) were cut with a Reichert microtome (Nussloch, FRG) and placed on chrome-gelatin-coated coverslips. Before staining, sections were rehydrated in xylene followed by decreasing concentrations of ethanol and finally distilled water, after which they were treated with 1% H₂O₂ in H₂O for 20 min. To prevent nonspecific binding of the antibodies, the sections were incubated in normal goat serum diluted 1/10 in PBS containing 4% (w/v) BSA for 15 min. Myrosinase was detected by 1 h incubation at room temperature with the monoclonal antibody 3D7, diluted 1/400 in PBS, followed by rabbit anti-mouse IgG antiserum, diluted 1/40 (DAKO-
Figure 2. Visualization of myrosinase in cells of a 39-d-old embryo of rapeseed. Consecutive sections were used for staining with a monoclonal antibody, 3D7, directed against myrosinase (a) and with Millon's reagent (b). The same cells were stained by both methods (arrows). The staining of the cytoplasm with the antibodies and the myrosin grains with Millon's reagent is illustrated at higher magnification in (c) and (d), respectively. For (a) and (b) scale bar equals 10 μm, for (c) and (d) scale bar equals 1 μm.
PATTS a/s, Copenhagen) in PBS containing 4% BSA, for 30 min. Finally, the sections were incubated with a complex of horseradish peroxidase and mouse antiperoxidase antibodies, diluted 1/250 in PBS with 4% BSA, for 30 min followed by development in 0.8 mm 3-amino-9-ethyl carbazole, 5% DMSO, 0.02 m sodium acetate (pH 5.0), to which 30% H₂O₂ was added to a final concentration of 0.05% (v/v). When the polyclonal antiserum K505 was used, it was diluted 1/12,000 in PBS. Goat anti-rabbit IgG antiserum, diluted 1/40 in PBS with 4% BSA, was employed as the secondary antibody. The immunocomplexes were detected by horse peroxidase and rabbit antiperoxidase antibodies followed by development as described above. For counter staining, Mayers HTX was used followed by a 10 min rinse in water. Specimens were mounted in glycine-gelatin and micrographs were taken in a Nikon optiphot.

In control experiments, the staining procedure was carried out as above but with the 3D7 antibody either replaced with a monoclonal antibody against an irrelevant antigen (human β₂-microglobulin) or pre-absorbed with excessive amounts of purified myrosinase prior to being applied to the specimens. For the polyclonal rabbit antiserum (K505), pre-immune serum was used.

Histochemical Staining of Myrosin Cells

Myrosin cells were identified in rapeseed embryos by staining with Millon’s reagent as described (7).

Western Analysis

Proteins were separated by electrophoresis in 10 to 15% polyacrylamide gradient gel containing SDS (1) and subsequently electrotransferred to nitrocellulose membranes (17). The filter was stained according to the protocols for the Protoblot system (Promega Biotec, Madison, WI). 3D7 was used as the primary antibody.

RESULTS

Immunolocalization of myrosinase in sections of Brassica napus embryos using either the polyclonal antiserum K505 or the monoclonal antibody 3D7 showed that the enzyme was present in only a small number of cells (Fig. 1a, c). No reactivity was seen when K505 was replaced with normal rabbit serum (Fig. 1b), or when 3D7 had been exchanged for a monoclonal antibody towards a human membrane protein (Fig. 1d) or preabsorbed with purified myrosinase (not shown). Since the staining with 3D7 gave a higher reproducibility, this reagent was used throughout this study.

The distribution of myrosinase as seen by immunohistochemistry was compared to that observed when the traditional technique for detecting myrosin cells, the Millon’s reagent, was used on consecutive sections of a rapeseed embryo taken 39 d after pollination. Evidently, the two procedures identified the same set of cells (Fig. 2). The antibody detected the enzyme either uniformly distributed in the cytoplasm or organized as particulate bodies. In addition, marked accumulation could be seen at the periphery of the myrosin grains (Fig. 2c).

To investigate the expression of myrosinase during embryogenesis, immature seeds were sampled at different times after pollination and stained for the enzyme. The results obtained by the immunolocalization technique showed that the enzyme accumulates during a relatively well defined period of the embryogenesis. The first myrosinase-containing cells appeared at approximately d 20, and they increased in number until approximately d 30, when 2 to 5% of the cells in each section were stained. At later stages of embryogenesis, the number of myrosin cells remained constant even though the staining became increasingly more intense (data not shown).

The synthesis of myrosinase during embryo growth was also analyzed by immunodetection with the 3D7 antibody on Western blots of protein extracts from seeds. According to these data, myrosinase appeared at d 30 after pollination and increased in amount to approximately d 40, after which it remained constant until the end of the ripening process (Fig. 3). A difference in sensitivity between the two methods may account for the somewhat later appearance of the enzyme when detected by immunoblotting as compared to the light microscopy.

In the seed, myrosinase was found solely in embryonic cells with a uniform distribution and no preference for either the cotyledons or the axis. The cells in the epidermis, meristematic cells in the axis, and the provascular cells in the cotyledon (not shown), however, did not appear to possess the enzyme. This distribution of myrosin cells in the embryo remained unchanged for the first 2 d after germination. The intracellular localization of the enzyme, however, changed toward a more pronounced accumulation around the vacuoles/myrosin grains during this period (Fig. 4a). Eight days after initiation of germination, the staining of the cotyledon revealed that myrosinase was expressed in the newly formed guard cells as well as in a small number of parenchyma cells (Fig. 4c). In the axis, a few elongated parenchyma cells were

![Figure 3. Western immunoblot analysis of myrosinase in total protein extracts from developing seeds sampled at different time points after pollination. Protein (50 μg) was loaded in each well. A 10 to 15% SDS-polyacrylamide gradient gel was used to separate the proteins. The figures above the lanes denote days after pollination. Molecular mass markers indicated are phosphorylase B (94 kD), bovine serum albumin (67 kD), and ovalbumin (43 kD).](image-url)
Presence of myrosinase in seeds during germination as visualized by staining with the monoclonal antibody 3D7. In panel (a) a high magnification of a cotyledon cell 2 d after initiation of germination, myrosinase appeared to be accumulated around the vacuoles. Panels (b) and (c) show the embryo 8 d after initiation of germination: in (b) part of axis with two positive parenchyma cells and some positive vascular cells, in (c) part of a cotyledon with stained guard cells and a few stained parenchyma cells. Insets show some of these cells at higher magnification (arrows in low magnification). Bar in low magnifications equals 10 μm, in high magnifications 1 μm.
Figure 5. Localization of myrosinase in leaf, petal, and siliquae of the plant using the monoclonal antibody 3D7. Panel (a) shows part of a foliage leaf with stained guard cells and parenchyma cells in the vascular tissue. Panels (b) and (c) are parts of a petal and a siliqua, respectively, with stained parenchyma cells in the vascular tissue. Bar in low magnifications equals 10 μm, in high magnifications 1 μm.
Figure 6. Localization of myrosinase in stem and root using 3D7. Panel (a) shows part of the stem with stained cells in the xylem as well as in the cortex. In (b) a section of a root with the major staining localized to the cortex region. The peripheral, rim-like staining was also observed in control specimens and is thus unspecific (data not shown). Bar in low magnifications equals 10 μm, in high magnifications 1 μm.
stained, and the enzyme also began to appear in the differentiating vascular tissue (Fig. 4b).

The staining pattern of young foliage leaves resembled that of the cotyledon with myrosinase being present in the guard cells and parenchyma. In addition, stained cells appeared in the vascular tissue close to the phloem (Fig. 5a). In older leaves, however, the guard cells no longer contained myrosinase. In petals and siliques, the localization of myrosinase was similar to that in the older leaves, i.e. scattered parenchyma cells were stained in the mesophyll as well as in the vascular tissue (Fig. 5, b and c). In the stem, a major location for the enzyme was the xylem cells, although some cells in the cortex also contained myrosinase (Fig. 6a). In the root the myrosin cells were mainly located in the cortex (Fig. 6b).

**DISCUSSION**

This paper presents the distribution of myrosinase studied by immunohistochemical staining of tissue sections. In preliminary studies, a polyclonal antiserum (K505) and a monoclonal antibody (3D7) were compared. Western blot analyses and immunoprecipitations had shown that these antibodies reacted with the same myrosinase species (9). The two reagents reacted in a similar way also when used in immunohistochemistry. Only a small number of cells were stained. Since K505 in quantitative immunoprecipitations reacted with more than 80% of all myrosinase activity present in a rapeseed protein extract (9), it is likely that very little if any myrosinase is located outside these cells. These results are in complete agreement with those of a recent study in which myrosinase was identified in *Brassica napus* seeds with a different monoclonal antibody (16). In a previous study, myrosinase appeared to be expressed in the majority of rapeseed embryonic cells (12), a suggestion in conflict with that of the present study. It seems reasonable that diffusion of the enzyme and/or the salts in the unfixed tissue of the earlier study might have caused an erroneous result.

Approximately 2 to 5% of the cells in the mature seed appeared to contain myrosinase. The first myrosinase-containing cells emerged at approximately d 20 after pollination. The number increased until approximately d 30. The more intense staining of these cells during the later stages of seed development suggested that the cells continued to accumulate myrosinase after this period. Indeed, when protein extracts from developing seeds were investigated for myrosinase by Western analysis, increasing amounts of the enzyme were recorded up to d 38 after pollination.

The confinement of myrosinase to only a few cells, and to different cell types in the various organs of the plant, indicates that a sophisticated regulatory mechanism governs the expression of the myrosinase genes. It follows that the promoter regions of these genes will be interesting to investigate with respect to the regulation of their cellular specificity.

In a comparative study on consecutive sections of seeds, we showed that Millon’s reagent and the antibodies identified the same set of cells. Thus, the immunocytochemical technique is a reliable method for the identification of myrosin cells. However, the two different techniques stain different compartments of the myrosin cells; the antibodies localize the enzyme in the cytoplasm, whereas Millon’s reagent stained the interior of the myrosin grains. The Millon’s reagent reacts with tyrosine and tryptophan residues, i.e. compounds which contain an aromatic ring structure (7). Some of the glucosinolates, the methylinolides, have structures similar to tryptophan and, hence, are probably the reactants detected by the Millon’s reagent. The localization of glucosinolates in the interior of myrosin grains is also in agreement with earlier findings that glucosinolates are present only in the myrosin cells where they are stored in the myrosin grains/vacuoles (3).

There are many conflicting reports concerning the intracellular localization of myrosinase; in early studies mainly based on morphological criteria, myrosinase was suggested to be localized to the interior of the myrosin grains (13, 18, 19), while results of later reports suggested a cytoplasmic distribution, sometimes in association with membranes (6, 12). The latter alternative is in accordance with our findings, which include an occasional association with the outside of the myrosin grains. The nature of the interaction of the enzyme with membranous structures is unclear, and elucidation of this question has to await further biochemical characterization of the myrosinase molecule.

The formation of a series of potentially toxic cleavage products upon the hydrolysis of glucosinolates by myrosinase has led to a proposal that the myrosinase-glucosinolate system might have a role as a nonspecific defense system in the plant (11). Our findings support this proposal, because we have shown that there is specific expression of myrosinase in guard cells and in xylem, which are both prime locations for pathogen invasion. It remains to be determined whether myrosinase synthesis might be induced in higher amounts by the addition of different agents that damage plant tissue.

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**LITERATURE CITED**

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