# Myrosinases, glucosinolates and transformation products in *Brassica*, *Raphanus* and *Sinapis*: Physico-chemical properties and activity of myrosinase isoenzymes

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## ABSTRACT

Myrosinases are glycoproteins composed by several minor protein subunits that occur as mixtures of isoenzymes. Myrosinases catalyse the hydrolysis of glucosinolates, producing a series of degradation products with biological activity. In the experiments presented in this paper, myrosinases were purified from seeds of *S. alba*, *B. napus* and two cultivars of *R. sativus* with different resistance to nematodes (Pegletta and Siletina). Three different molecular weight groups (500-600 kD (III), 280-350 kD (II) and 140-190 kD (I)) were found by G-200 gel filtration for *B. napus* and *R. sativus* myrosinases, whereas *S. alba* only showed the group of isoenzymes with the lowest molecular weight (I). By SDS-PAGE it was found that all isoenzyme groups were composed by 8-10 subunits of molecular weight ranging from 20-80 kD. In an assay with sinigrin the three isoenzyme groups within a single species showed different activity, the group with the lowest molecular weight (I) showing the lowest activity in both *B. napus* and *R. sativus*. The different activity against sinigrin showed by both cultivars of *R. sativus* may be related to their different resistance against nematodes although other mechanisms are also likely to be involved. The possibility for utilising Brassicaceous plants for their biological activity (fungicidal, nematocidal, nutritional, anticarcinogenic, etc…) requires the determination of the glucosinolate content of the original material, but it also requires the careful study of the myrosinase isoenzymes and the products resulting from their action on glucosinolates.

**KEYWORDS: Myrosinases; isoenzymes; glucosinolates; biological activity; Brassicaceae.**

## INTRODUCTION

Myrosinases (EC 3.2.1.147) occur as mixtures of different isoenzymes in all glucosinolate-containing plants (*1*). The active and native isoenzymes are glycoproteins composed by several minor protein subunits, associated in membrane bound oligomers, which are physically separated in the plant tissues from glucosinolates (*1-3*). With autolysis or damage of the plant tissues glucosinolates and myrosinases come into contact and in the presence of water, various types of glucosinolate derived products are formed. The type of products formed depends on the glucosinolate type, the type of myrosinases and the reaction conditions (*1, 4*).

Glucosinolate degradation products have traditionally been related to quality aspects of Brassicaceous crops used as feed and food (*1, 5*); however, interest in their biological activity (related to their fungicidal, nematocidal or anticarcinogenic properties) has increased in the last years (*6, 7*). A number of processes related to the biological activity of glucosinolate hydrolysis compounds (e. g. biofumigation, consumption of fresh

Brassicaceous vegetables, etc…) involve autolysis, which is the degradation of endogenous glucosinolates in disrupted plant material due to myrosinase-catalysed hydrolysis (*8, 9*).

Studies of the biological properties of glucosinolate containing plants have traditionally been based on the determination of the glucosinolate types and concentrations. Both of these parameters vary between plant genera, species, cultivars and even between plant parts and along development for a single plant (*10-12*). However, in autolysis and in processes and bioprocessing technologies developed for the production of high-quality oils, proteins and bioactive products from these plants (*9, 13*), the type of native myrosinases present in the starting material and the development of methods for its inactivation are of great importance. The possibility for utilising Brassicaceous plants for nutritional, health-beneficial and biofumigation purposes among others therefore requires the careful study both of glucosinolates and myrosinase isoenzymes in the species of interest.

Our present work has its focus on the above mentioned issues related to the types of glucosinolates and myrosinases present in the genera *Sinapis, Raphanus* and *Brassica*, as well as to the transformation products of glucosinolates and the possibility to control and utilise their bioactive properties. In this paper, the physico-chemical characteristics of myrosinases present in three species belonging to the above mentioned genera are determined. The activity of the myrosinase isoenzymes was also determined in an assay with sinigrin.

## MATERIALS AND METHODS

**Plant material.** Seeds of *B. napus* L. cv. Pollen, *S. alba* L. cv. Albatros, *R. sativus* L. cv. Pegletta (cultivar 80% resistant against *Heterodera schachtii*) and *R. sativus* L. cv. Siletina (susceptible cultivar), were used in the experiments.

**Isolation and purification of myrosinases.** *Preparation of crude extract.* Seeds (30-50 g) were thoroughly milled in a coffee grinder and defatted by Supercritical Fluid Extraction (Spe-ed SFE, Applied Separations, USA) with $CO_2$ (40 ºC, 600 bar, 45 min, 4 L $CO_2$/min). The defatted powder was water-extracted twice in an ice-bath with use of Ultra-Turrax homogenization.

*Purification process.* Isolation of myrosinase isoenzymes and determination of the molecular weight was performed respectively by Con-A affinity chromatography and Sephadex G-200 gel filtration with the use of a molecular weight standard (*14, 15*). The myrosinase activity and protein concentration of the fractions resulting from gel filtration were determined and fractions were pooled in groups accordingly (III, II, I, A, B). These groups were subsequently analysed for total protein concentration and myrosinase activity.

*Enzyme pI and subunit molecular weight.* Determination of isoenzymes pI and subunit molecular weight was performed by Isoelectric Focusing and SDS-PAGE respectively in a Pharmacia Phast System (*14, 15*).

**Enzymatic assay.** The activity of the myrosinase isoenzymes was determined by a spectroscopic assay with sinigrin (analytical grade from the laboratory collection) as a substrate. For all species, the assay was conducted in phosphate buffer (50 mM, pH 6.5). Ascorbic acid and sinigrin were added to the reaction to a final concentration of 0.25 mM and 0.2 mM respectively. One unit (U) of the enzyme catalyse the transformation of 1 μmol sinigrin ($E_{227nm}$=8000 $M^{-1}$ $cm^{-1}$) into products per minute (*15*).

**Protein determination.** Protein concentration was determined spectrophotometrically using Labert-Beers law and $E^{1\%}_{1cm}$=10 for all protein solutions (*15*).

## RESULTS AND DISCUSSION

**Purification of isoenzymes.** The *Brassica* and *Raphanus* myrosinases were separated by gel filtration into molecular weight (MW) groups of 500-600 kD (III), 280-350 kD (II) and 140-190 kD (I). Myrosinases from *S. alba* consisted only of isoenzymes with MW of 140-190 kD (I). Figure 1 shows the elution profile of the Con A myrosinase isolate of the four cultivars studied. Two protein peaks of a lower molecular weight (50-80 kD and a molecular weight value below the range of the column material) were observed for all four samples but they did not show any myrosinase activity (data not shown).

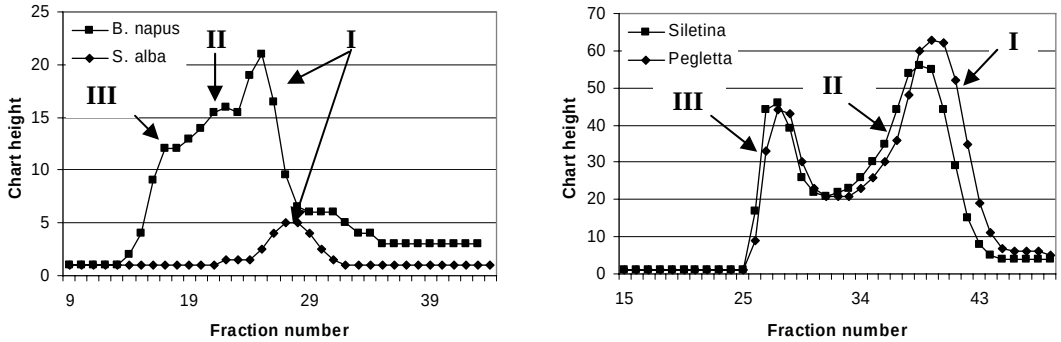


**Figure 1.** G-200 gel filtration elution of *B. napus* and *S. alba* (left) and *R. sativus* cv. Siletina and Pegletta (right). A molecular weight standard was used for determination of molecular weight ranges of the fractions (*15*).

Myrosinases have traditionally been described to have molecular weights ranging from 150 to 800 kD, depending on species and plant part studied (*2*). The isolation method can play an important role in the final determination of the molecular weight of myrosinase isoenzymes and whereas some authors have found myrosinase isoenzymes to be either dimmers, tetramers or hexammers of a single subunit (*16-19*), others have described myrosinase to be composed of many different molecular weight subunits giving rise to different molecular weight groups of isoenzymes (*14, 20*).

**Myrosinase activity and protein content.** Myrosinase activity varied depending on the isoenzyme group and the species considered (Table 1). Despite showing a similar protein concentration, myrosinase isoenzymes from the two *Raphanus* species showed marked differences in specific activity, which can be related to the different resistance that these cultivars show against nematodes. Quantitative and qualitative differences in glucosinolate concentration have been related to the different resistance of certain Brassicaceae species against nematodes (*21*), however, studies relating the myrosinase activity to the resistance of crop species to these herbivores are not frequent. Although there are probably other factors influencing this resistance, it is likely that the glucosinolate-myrosinase system plays an important role (*21, 22*).

Despite not being the major glucosinolate in any of the species studied, the activity that myrosinase isoenzymes from all four sources showed against sinigrin is quite high. For the two species showing three isoenzymes groups (*B. napus* and *R. sativus*), group I is the least active when compared to the other two groups. Myrosinase from *S. alba* (group I) was found to be more active than any of the three groups of *B. napus* or *R. sativus*.

**Table 1.** Protein content and activity of the myrosinase isoenzymes from the three species.

| | *B. napus* | | | *S. alba* | *R. sativus* Pegletta | | | *R. sativus* Siletina | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Isoenzyme group** | **III** | **II** | **I** | **I** | **III** | **II** | **I** | **III** | **II** | **I** |
| **Protein conc. (mg/mL)** | 0.06 | 0.12 | 0.12 | 0.11 | 0.31 | 0.20 | 0.45 | 0.32 | 0.15 | 0.40 |
| **Activity (U/ml)** | 0.90 | 1.13 | 0.53 | 2.89 | 5.85 | 1.43 | 2.96 | 3.75 | 3.08 | 1.91 |
| **Specific activity (U/mg)** | 14.75 | 9.78 | 4.53 | 26.49 | 19.06 | 7.13 | 6.58 | 11.83 | 21.06 | 4.73 |

**Enzyme pI and subunit molecular weight.** All groups of the species studied showed to be composed by a number of different isoenzymes of pI from 4.4 to 7.1 in agreement with previous results (*23*), although most of them were grouped around pH 4.8-5.6 (data not shown).

All myrosinase isoenzymes from the four different sources were composed by protein complexes formed by 8-12 subunits of different molecular weight (Figure 2). These subunits were present in different proportions depending on the isoenzyme group (I, II and III) and the species considered. The three molecular weight groups (I, II and III) were found to be quite similar when comparing the two *Raphanus* cultivars, although they differed from the three groups of *B. napus*, for which certain protein subunits were absent or present in different proportion. The only group of isoenzymes in *S. alba* (I) was found to be different from group I of the two *Raphanus* cultivars but was more similar to group I of *B. napus*.

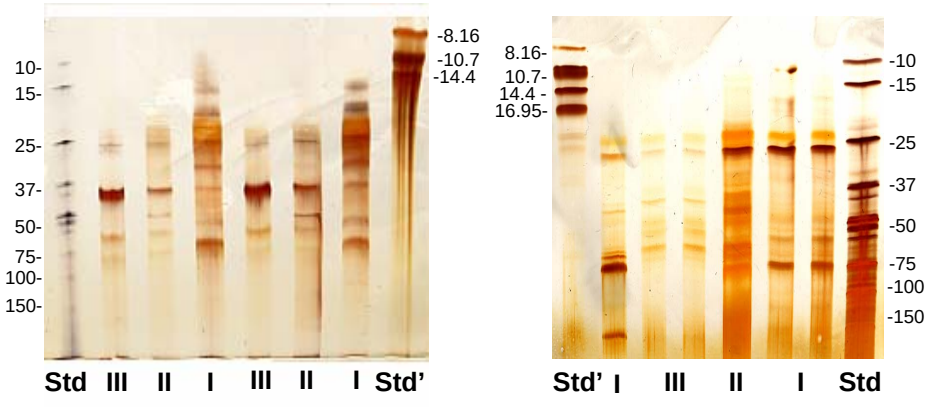


**Figure 2.** SDS-PAGE of myrosinase proteins in seeds of *R. sativus* cultivars Pegletta and Siletina (left) and *S. alba* and *B. napus* (right). Molecular weight of the standards (Std and Std') is indicated on both sides of the figure.

Despite being present in different proportions, bands at molecular weights of 20-25, 45-50 and 65-80 kD were present in all groups of isoenzymes of the species studied. These protein subunits may correspond to myrosinase isoenzymes, as coded by the gene families MA, MB and MC (*24*) along with a number of other associated proteins of molecular weights ranging from 30 to 100 kD, such as myrosinase binding proteins, myrosinase associated proteins and epithiospecifier proteins (*20, 25-27*).

## CONCLUSIONS

The different subunit composition of the myrosinase isoenzymes as indicated by SDS-PAGE along with the different pI profile (data not shown) indicates differences in the isoenzyme structure both between species but also between cultivars of a single species. These differences in structure may play a role in the different affinity that the isoenzymes have for the substrate, as shown by their activity against sinigrin.

The different structures of the isoenzymes could also influence the outcome of the glucosinolate hydrolysis with different types and quantities of degradation products being formed depending on the species or cultivar. The relative proportion in which the isoenzymes are present in different plant parts (*2, 28*) is also of relevance if we consider the isolation of myrosinase from different plant tissues or with respect to autolysis processes, such as biofumigation.

Further investigation of the products formed at different degradation conditions by native myrosinase isoenzymes of different origin is therefore needed before the use of concentrates, isolates or extracts from these plants for nutritional, health-benefit and/or plant protection purposes is implemented.

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