MYROSINASES IN BRASSICA: CHARACTERIZATION AND PROPERTIES

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INTRODUCTION

Myrosinases (β-thioglucoside glucohydrolase; EC 3.2.3.1) are present in all plants containing glucosinolates (Buchwaldt et al., 1986). Myrosinases catalyze the hydrolysis of the thioglucoside bond in glucosinolates, thereby producing D-glucose, sulphate, and various amounts of different products depending on hydrolysis conditions (Olsen and Sørensen, 1981). Myrosinases exist as isoenzymes in different numbers depending on plant source and developmental stage, and reports concerning number of isoenzymes are not always in agreement with each other (Buchwaldt et al., 1986). Myrosinases are glycoproteins, and rapeseed has been found to contain at least four isoenzymes with small differences in molecular weight and isoelectric points (Björkman and Lönnerdal, 1973; Lönnerdal and Janson, 1973; Buchwaldt et al., 1986; Bones and Slupphaug, 1989). Investigations of enzymatic properties of different isoenzymes have included pH optima, temperature optima, and ascorbic acid optima for mixtures of isoenzymes or single isoenzymes (Björkman and Lönnerdal, 1973; Lönnerdal and Janson, 1973; Bones and Slupphaug, 1989) but not kinetic parameters for different glucosinolates and single isoenzymes. Common to all previous investigations on myrosinases are, that only a part of the separated proteins with myrosinase activity have been investigated.

The aim of the present investigations has been directed at solution of the unsolved problems and determination of the properties of individual myrosinase isoenzymes including their catalytic effects towards structurally different glucosinolates.

MATERIALS AND METHODS

Crude Extracts

Seeds of Brassica napus were ground and extracted with water (15% w/v) by use of an Ultra Turrax homogenizer (3x5 min, 2°C), extracted (2 min, 2°C) after addition of $CHCl_3$ (33% v/v) and centrifuged (15000 rpm, 30 min, 4°C) giving a clear aqueous crude extract.

Affinity Chromatography

Crude extracts were loaded on Con-A Sepharose columns (Pharmacia) and washed with buffer A (50 mM phosphate, pH 6.8 containing 0.15 M NaCl) until absorbance at 280 nm was zero. Myrosinase was eluted with either water or buffer A containing 0.5 M methyl-α-D-mannopyranoside (Palmieri et al., 1986). After each fraction the flow was stopped for 30 min.

FPLC Chromatofocusing

Samples from affinity chromatography and gelfiltration were applied to a prepacked Mono P HR 5/20 column (Pharmacia) equilibrated with buffer B (20 mM bis-Tris, pH 7.1). The column was eluted with 3 ml buffer B, 46 ml buffer C (10% Polybuffer 74 Pharmacia, pH 4.0) and 3 ml buffer B.

Gel Filtration

Samples from affinity chromatography and FPLC chromatofocusing were gel filtrated on a Sephadex G-200 (55x1000 mm) column (Pharmacia) using buffer A.

Isoelectric Focusing (IEF)

Gels for isoelectric focusing (20.5x11.0 cm; A: T 10.3%; C 2.6% and B: T 5.0%; C 13.1%) containing 7.1% Ampholine were chemically polymerized using TEMED and ammoniumpersulphate. Phast System (Pharmacia) isoelectric focusing and gradient gel electrophoresis were also performed using pre-casted or lab-casted gels.

Immunotechniques

Antibody production and isolation were performed according to Harboe and Ingild (1973). Rocket immunoelectrophoresis was modified from Weeke (1973).

Protein Determination

Protein content was determined by measuring absorbance at 280 nm using an $A_{1cm}^{1\%}$ value of 10. Protein bands in gels were stained with silver according to Switzer et al. (1979).

Myrosinase Assay

Myrosinase activity was measured by a direct spectrophotometric assay (modified from Schwimmer, 1961), where decrease in absorbance at 227 nm resulting from hydrolysis of sinigrin was recorded. One unit was defined as the amount of enzyme activity that hydrolysed 1 μmol of sinigrin per minute at 30°C in buffer E (30 mM phosphate, pH 6.5 containing 0.4 mM sinigrin and 0.5 mM ascorbic acid).

Myrosinase Spottests

Detection of myrosinase in gels after electrophoresis was performed by placing gels in a solution A of 1 mg sinigrin, 5 mg barium chloride, 0.5 mg ascorbic acid and 0.1 ml acetic acid per millilitre of solution or a solution B of 50 mM citrate buffer containing 1 mg sinigrin, 5 mg barium chloride, and 0.5 mg ascorbic acid per millilitre buffer and adjusted to pH 6.5. For detection of myrosinase in different fractions 50 μl of myrosinase fraction was mixed with 50 μl sinigrin solution (15 mM), reaction for 1 hour and addition of 20 μl glucose assay reagent (Glucose 115 procedure Sigma). Myrosinase activity gave a red colour after 10-20 min.

RESULTS

Extraction was performed on seed samples of different cultivars of Brassica napus, and affinity chromatography was performed in Con-A columns. Results obtained from extraction of myrosinase from Brassica napus cv. Doral gave a recovery of 97.0% of activity. The extraction procedure with $CHCl_3$ gave crude extracts with an activity as found for extraction in water without $CHCl_3$, but the crude extracts after $CHCl_3$ extraction gave much easier and faster separations on Con-A columns.

Gelfiltrations were performed on Con-A fractions. Gelfiltration on fractions 1-8 are shown in Fig. 1. Enzyme form T_1 accounted for 46%, T_2 for 30% and D for 23% of the total activity recovered, and their specific activities were 18.5 U/mg, 10.8 U/mg, and 3,9 U/mg, respectively. Molecular weight estimations of the enzyme forms were 550 kD, 280 kD, and 150 kD using standards ranging from 670 to 17 kD.

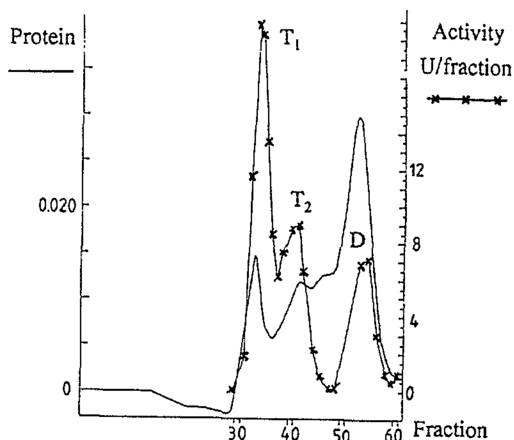


Fig. 1. Gelfiltration of myrosinase fraction 1-8 from Con-A Sepharose (Exp. A) on Sephadex G-200 (55x1000 mm) using buffer A. (—) protein (A_{276}), (x—x) myrosinase activity.

FPLC chromatofocusing of Con-A fractions from B. napus cv. Line (Exp. B) showed activity eluted according to pH values from 5.67 to 4.5 (Fig. 2) indicating several isoenzymes with different pI values.

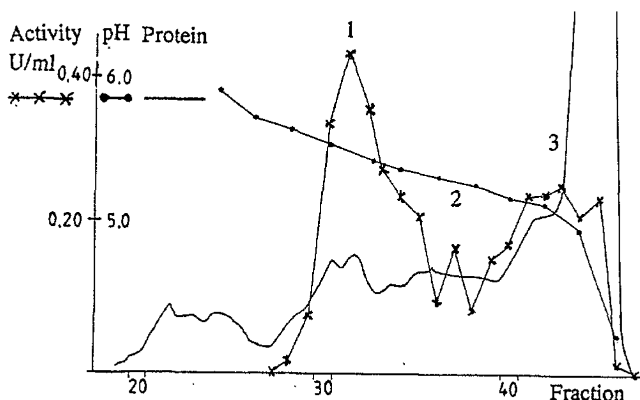


Fig. 2. FPLC chromatofocusing of Con-A fractions from B. napus cv. Line (Exp. B). (—) protein, (x—x) myrosinase activity, (•—•) pH.

The activity appeared in two major areas (fraction 28-36 (1) and 38-46 (3)) and one minor area (fraction 36-38 (2)) accounting for 52.2%, 41.0%, and 6.8% respectively of total activity recovered, and total recovery of activity was 73%. Myrosinases in the first area appeared as three protein peaks (IH, IM, and IL).

Results obtained from FPLC chromatofocusing of the enzyme forms T_1, T_2, and D are shown in Fig. 3. D eluted at pH 5.6-5.2, T_2 at pH 5.5-4.1, and T_1 at pH 5.2-4.0, and the chromatograms show that there are more than three enzyme forms with different pI values. The recovery of T_1 activity was low compared to T_2 and D, indicating that T_1 is more affected than the other enzyme forms by chromatofocusing.

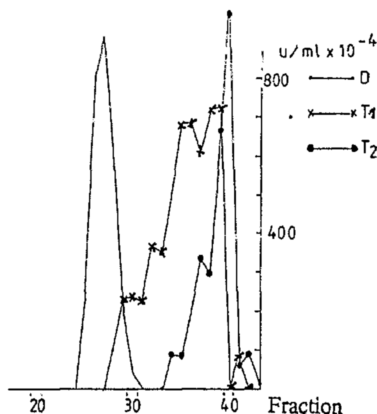
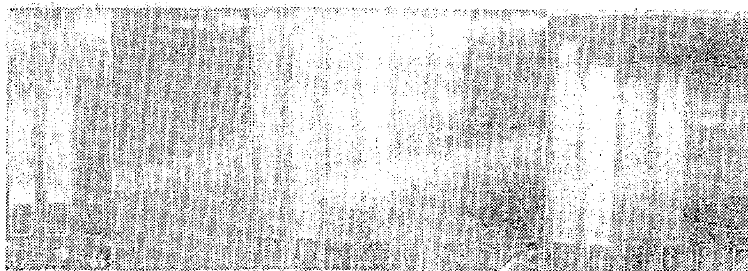


Fig. 3. FPLC chromatofocusing of T_1, T_2, and D from gelfiltration on Sephadex G-200. (x—x) myrosinase activity T_1, (•—•) myrosinase activity T_2, (—) myrosinase activity D.

Isoelectric focusing (IEF) of different enzyme fractions is shown in Fig. 4. It was found that enzyme forms T_1 and T_2 were too large to migrate and focus in gel A, whereas this was possible in gel B. It was also found, that spottest with the traditionally used solution A only gave reaction with enzyme form D, whereas solution B made it possible also to see enzyme forms T_1 and T_2. This suggests that enzyme forms T_1 and T_2 have a lower activity or are inactive at the low pH of solution A. Enzyme form D contained 3 isoenzymes with pI values 5.00, 5.15, and 5.28 (IL, IM, and IH). T_1 and T_2 consisted of isoenzymes with pI's in at least 5 pH regions (pH 5.2, 5.3, 5.5, 6.1, and 6.2). Gradient gel electrophoresis (8-25% and 10-15%) using Phast System confirmed that the three enzyme forms T_1, T_2, and D had different sizes, and T_1 was not even able to migrate out of the stacking gel.



<table>
<tr><td>Enz.</td><td>T₁</td><td>T₂</td><td>D</td><td>T₂</td><td>T₁</td><td>D</td><td>D</td><td>Con-A</td><td>T₁</td><td>T₁</td><td>T₂</td><td>T₂</td><td>D</td><td>D</td><td>T₁</td><td>T₁</td><td>T₂</td><td>T₂</td><td>D</td><td>D</td></tr>
<tr><td>Gel,Spot</td><td colspan="3">A,B</td><td colspan="4">B,A</td><td colspan="13">B,B</td></tr>
<tr><td>pH</td><td colspan="14">3.5-9.5</td><td colspan="6">4.0-7.0</td></tr>
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Fig. 4. Isoelectric focusing of different enzyme fractions (Con-A, T_1, T_2, and D), gel composition (A and B), pH (3.5-9.5 and 4.0-7.0), and spottests (A and B).

Individual myrosinase isoenzymes (IH, IM, and IL; Fig. 2) have been purified by FPLC chromatofocusing and checked by IEF developed for protein and enzyme activity. Kinetic properties of IH, IM, and IL have been investigated using various glucosinolates as substrates. Some results are presented in Fig. 5. K_m and V_{max} varied depending on

isoenzyme and substrate showing that different isoenzymes have different kinetic properties with the same substrate, and that the same isoenzyme has different kinetic properties with different substrates. Investigations using desulfoglucosinolates and glucosinolates with a substituted glucose unit as substrates showed that the sulphate group and the unsubstituted glucose unit in glucosinolates are necessary for enzymatic hydrolysis to occur, but some weak esterase activity - without myrosinase activity, was observed.

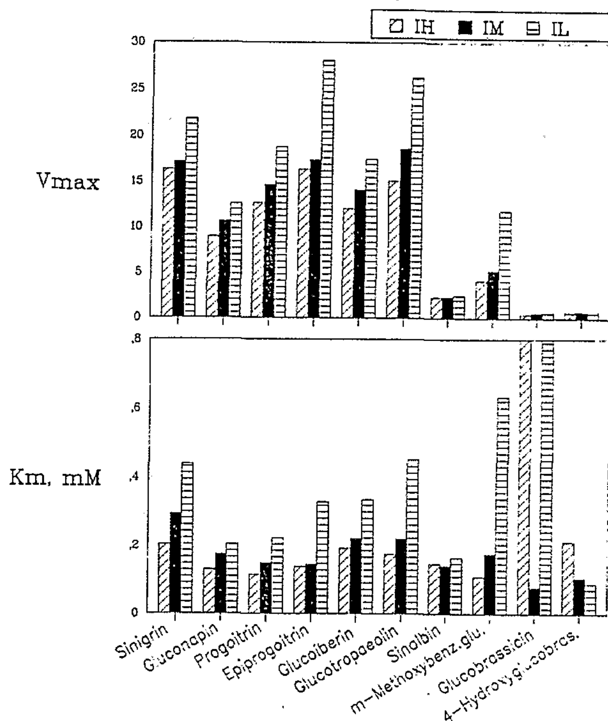

Fig. 5. Kinetic properties of three purified isoenzymes: IH, IM, and IL (MW : 150 kD) tested against 10 different glucosinolates.

Antibodies were produced against Con-A myrosinase and the isoenzyme IL. Rocket immunoelectrophoresis of Con-A myrosinase, T_1, T_2, IL, IM, and IH gave each 2-6 precipitation rockets of varying height indicating the existence of up to 20 or more immunochemically different enzyme forms of myrosinase.

DISCUSSION AND CONCLUSION

Affinity chromatography gave nearly 100% recovery of activity and the new extraction procedure with $CHCl_3$ gave easier Con-A separations. Myrosinase from B. napus consisted of three enzyme forms T_1, T_2, and D with molecular weights of 550, 280, and 150 kD. T_1 and T_2 represented 76% of the activity, but these forms have apparently not yet been described in detail in literature. FPLC chromatofocusing of Con-A myrosinase showed 2 major areas and 1 minor area with activity. Chromatofocusing of T_1, T_2, and D revealed that they are eluted in the order D, T_2, and T_1, with decreasing pH. IEF gave pI values of 5.00, 5.15, and 5.28 for IL, IM, and IH which corresponds well with myrosinase "C" investigated by Lönnerdal and Janson (1973) and Bones and

This could be due to delayed elution because of the larger size of T_1 and T_2 compared to D. The lower recovery of T_1 from chromatofocusing suggests that especially T_1 is affected by chromatofocusing conditions. Isoelectric points of T_1 and T_2 in the same pH region as D indicates that IEF purity analysis is not sufficient for distinguishing between the three size groups of different isoenzymes T_1, T_2, and D. Isoelectric points of T_1 and T_2 suggest that they could contain the enzymes called "A", "B" and/or "D" by Lönnerdal and Janson (1973). The different kinetic properties of isoenzymes towards the same or different glucosinolates indicate the need for investigations on glucosinolate degradation products as a result of hydrolysis with different myrosinase enzyme forms. Immunochemical investigations together with the other investigations suggest the existence of many more isoenzymes than previously reported. Further investigations of the physical, enzymatic, and immunochemical properties of myrosinase are now expected to provide more complete informations, which are considered important in relation to improved quality of rapeseed and rapeseed products.

ACKNOWLEDGEMENT

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REFERENCES

BJÖRKMAN, R. and LÖNNERDAL, B. 1973. Studies on myrosinases. III. Enzymatic properties of myrosinases from Sinapis alba and Brassica napus seeds. Biochim. Biophys. Acta 327, 121-131.

BONES, A. and SLUPPHAUG, G. 1989. Purification, Characterization, and Partial Amino Acid Sequencing of β-thioglucosidase from Brassica napus L. J. Plant Physiol. 134, 722-729.

BUCHWALDT, L., LARSEN, L.M., PLÖGER, A. and SØRENSEN, H. 1986. Fast Polymer Liquid Chromatography isolation and characterization of plant myrosinase, β-thioglucoside glucohydrolase, isoenzymes. J. Chromatogr. 363, 71-80.

HARBOE, N. and INGILD, A. 1973. Immunization, isolation of immunoglobins, estimation of antibody titre. Chapter 23 in: A manual of quantitative immunoelectrophoresis methods and applications (Eds. Axelsen, Krøll and Weeke). Universitetsforlaget, Oslo.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680.

LÖNNERDAL, B. and JANSON, J.-C. 1973. Studies on myrosinases. II. Purification and characterization of a myrosinase from rapeseed (Brassica napus L.). Biochim. Biophys. Acta 315, 421-429.

OLSEN, O. and SØRENSEN, H. 1981. Recent advances in the analysis of glucosinolates. J. Am. Oil Chem. Soc. 58, 857-865.

PALMIERI, S., IORI, R. and LEONI, O. 1986. Myrosinase from Sinapis alba L.: A new method of purification for glucosinolate analyses. J. Agric. Food Chem. 34, 138-140.

SCHWIMMER, S. 1961. Spectral changes during the action of myrosinase on sinigrin. Acta Chem. Scand. 45, 535-544.

SWITZER, R.C., MERRIL, C.R. and SHIFRIN, S. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Anal. Biochem. 98, 231-237.

WEEKE, B. 1973. Rocket immunoelectrophoresis. Chapter 2 in: A manual of quantitative immunoelectrophoresis methods and applications (Eds. Axelsen, Krøll and Weeke). Universitetsforlaget, Oslo.