PREPARATIVE PURIFICATION OF MYROSINASE FROM SINAPIS ALBA L. - CHARACTERIZATION OF POLYCLONAL ANTIBODIES AGAINST MYROSINASE.

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INTRODUCTION

Myrosinase (β-thioglucosidase, thioglucoside glucohydrolase, E.C. 3.2.3.1) is a group of enzymes primarly occurring in species from Brassicaceae. They hydrolyze glucosinolates, a group of thioglucosides, thereby producing toxic compounds such as nitriles and isothiocyanates (Fenwick et al., 1983). The function of the myrosinase-glucosinolate system in plants is not completely known, but it is suggested that it has an important role in the defence system against microorganisms (Chew, 1988).

In a recent paper the purification, characterization and partial amino acid sequence of myrosinase from seeds of Brassica napus L. was reported (Bones and Slupphaug, 1989). This purification method included three main steps; affinity chromatography on concanavallin A coupled to agarose, ion exchange chromatography on DEAE-cellulose and fast protein liquid chromatography on a Mono Q column. Myrosinase from Sinapis alba L. has been purified and characterized by Björkman and Janson (1972). Their method included time consuming steps, e.g. anion exchange chromatography and rechromatography with a linear ionic strength gradient and gelfiltration on a Sephadex 200 column.

In this new method we have replaced this and other multicolumn systems with two simple preparative steps, consisting of an initial affinity chromatography followed by preparative isoelectric focusing. Compared with earlier methods, this new method is much faster, gives higher yields of enzyme and is much easier to perform.

The purified protein has been used to raise rabbit polyclonal antibodies against myrosinase.

MATERIALS AND METHODS

Preparation of Crude Extract and Affinity Chromatograpy.

Crude dialyzed extract (320 ml) from 50 g seeds from Sinapis alba L. cv Trico (white mustard) was loaded on a con A-Sepharose (Pharmacia) column (Palmieri et al., 1986) using the method described by Bones and Slupphaug (1987,1989). A total of 90 ml eluate was collected and dialyzed against 4 mM imidazole-HCl buffer (pH 6.0).

Preparative Isolelectric Focusing

Preparative isoelectric focusing was performed with a Rotofor™ preparative IEF cell (Bio-Rad). A solution consisting of 50 ml con A-Sepharose eluate and 2% (v/v) BioLyte 4-6 (BioRad) was mixed and loaded into the cell through a syringe. The cell was sealed and run at constant power (12 W) for approximately 200 Vh. Samples (approximately 2 ml) were harvested with a vacuum harvesting system (Bio-Rad). After determination of specific activity, the two fractions with highest specific myrosinase activity were collected, diluted with distilled water to 50 ml, and refractionated without addition of more BioLyte solution. The experiments were run at 4°C and constant temperature maintained by using a GenLine™ cooler and GenLine™ heat exchanger (Beckman) fitted to the IEF cell.

Electrophoresis and Isoelectric Focusing in Polyacrylamide Gels

Polyacrylamide gel electrophoresis (PAGE) was performed both with intact myrosinase (native-PAGE) and after reduction and denaturation (SDS-PAGE). Electrophoresis was performed in gradient gels or in homogeneous gel. The protein

markers used were the high molecular weight standard mixture (Pharmacia). Isoelectric focusing (IEF) was performed in homogeneous polyacrylamide gels (5% T, 3% C) containing Pharmalyte (Pharmacia) carrier ampholytes generating a linear pH gradient from 4-6.5. The markers used were the isoelectric focusing calibration mixture (Pharmacia). Gels were silver stained according to Heukeshoven and Dernick (1985).

Myrosinase and Protein Assay

Myrosinase was measured as described by Bones and Slupphaug (1989). Protein was measured with the Bio-Rad protein assay using bovine serum albumin standards.

Production of Polyclonal Antibodies

Highly purified myrosinase (150 µg) in 750 µl of PBS (phosphate buffered saline) was emulsified with an equal amount of Freund's complete adjuvant and was injected into Chinchilla rabbits (Dr. Carl Thomae GmbH, Federal Republic of Germany). One ml mixture was administered intradermally at about 20 sites and the rest (500 µl) was injected subcutaneously. Booster injections of 100 µg myrosinase 1:1 with Freund's incomplete adjuvant were administered subcutaneously at about 3 weeks intervals. Blood was collected from the marginal ear vein. After removal of clots, the serum was clarified and lipids removed using trichlortrifluorethan (Frigen, Hoechst)(1.5 vol serum : 1 vol. Frigen). After vortexing for 1 min the mixture was centrifuged at 1000 xg for 10 min. Serum in the upper phase was divided into aliquots and stored frozen at -70°C.

Immunogold labelling

Fixation, sectioning and labelling were performed as described by Thangstad et al. (1991).

RESULTS AND DISCUSSION

Purification of Myrosinase

The purification by con A-Sepharose was a combination of column chromatography and batch eluation. The advantage of this combination was that the sample could be loaded and washed under strictly controlled conditions and at the same time be eluated from the matrix in a small volume of buffer. The eluation was performed at room temperature due to a shorter eluation period. After batch eluation the specific activity was approximately 8-fold higher than the dialyzed and centrifuged crude extract (Tab. 1).

Table 1: Purification of myrosinase from 50 g Sinapis alba seed.

Step	Protein (mg)	Tot. act. (µmol min^{-1})	Spec. act. (µmol $min^{-1}mg^{-1}$)	Yield (%)	Purification (fold)
Crude dialyzed extract	284	1447	5.09	100	-
Con A-Seph.	23.1	922.5	39.99	63.7	7.9
Rotofor[a)]	4.21	737.6	174.97	51.0	34.4

a) Sum for three repetitive runs.

Preparative Isoelectric Focusing

As many of the purification protocols for myrosinase include at least one step with anion-exchange chromatography, usually DEAE-cellulose, which is tedious and

time consuming due to the gradient eluation, we tried several methods to by-pass this step. Of these only preparative isoelectric focusing could replace the ion-exchange chromatography and at the same time give a higher purification. Figure 1 shows the results from fractionation of con A-Sepharose eluate by isoelectric focusing in the Rotofor IEF cell. As can be seen from the gels and from the profile given in Fig. 1, fraction 13 contains nearly all myrosinase activity and a very small amount of contaminating proteins. By refractionation of the two fractions with highest specific myrosinase activity, apparently homogeneous myrosinase was obtained (Fig. 2) at a yield (>50%) approximately 2.5 times higher than reported before (Björkman and Janson, 1972). The quality of this myrosinase was examined by isoelectric focusing in polyacrylamide gels, SDS-PAGE and native-PAGE. Cyanogen bromide cleavage and amino acid sequencing also supported that the enzyme preparation was apparently homogeneous (data not shown). Furthermore, the high specific activity strongly indicates that the enzyme was highly purified. In other experiments larger amounts of protein were loaded on the Rotofor cell. An assay of purity using FPLC and a monoQ anion-exchange column in combination with SDS-PAGE, native-PAGE and IEF, showed that minor amounts of impurities were present. These impurities could be removed in this step.

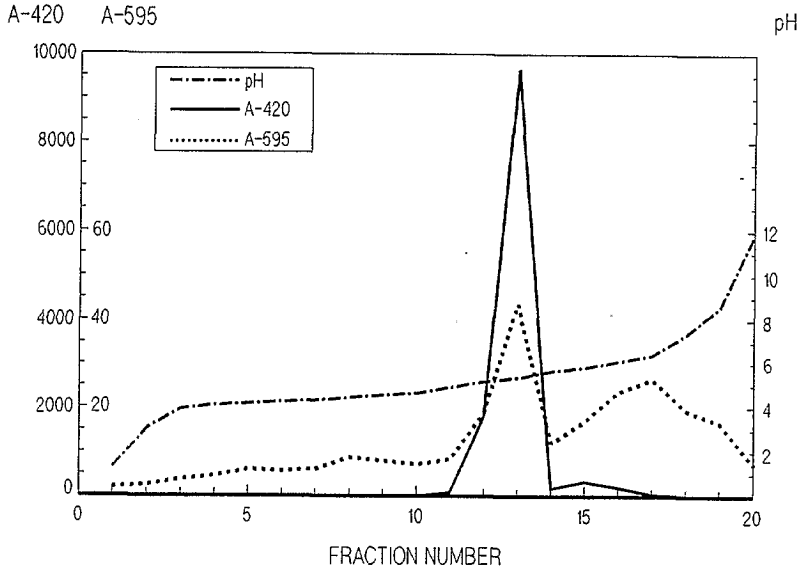

Fig. 1. Preparative isoelectric focusing of eluate after con A-Sepharose. Most of the myrosinase activity can be found in fraction 13. Myrosinase activity (A420), protein (A595) and pH in each fraction is indicated.

Production and characterization of polyclonal antibodies

Homogeneous myrosinase controlled by FPLC was used for immunization of rabbits. Sera obtained after two immunizations showed high binding activity against myrosinase. The antibody was shown to react specifically in enzyme-linked-immunosorbent-assay (ELISA) at a dilution up to 1:500 000 (Fig. 3). Western blots after both SDS-PAGE and isoelectric focusing using both crude extracts and purified myrosinase confirmed the specificity (Fig. 4). The sensitivity of the antiserum was on immuno-dot-blots and slot-blots as low as 0.25 pg purified myrosinase. In a test of crude and purified myrosinase from eight species within the Brassicaceae the antiserum detected myrosinases in all species, although with a slightly variable sensitivity (data not shown). Detection of myrosinase was also confirmed by immunogold-EM localization of myrosinase in myrosin cells of four

species of Brassicaceae (Thangstad et al., 1991). Examples of the immunogold localization of myrosinase are shown on Fig. 5.

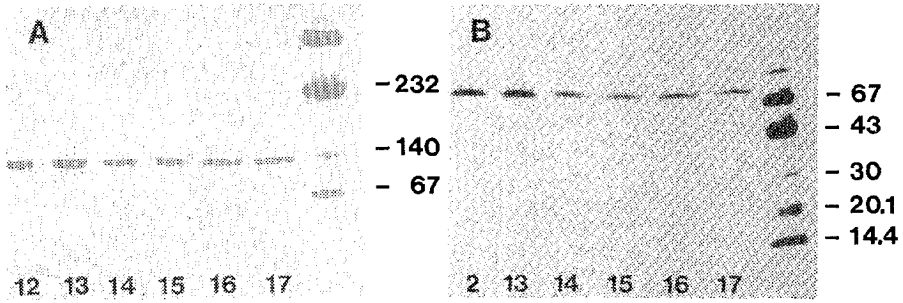

Fig. 2. (A) Native- and (B) SDS-PAGE of fraction 12-17 after rechromatography of the two best fractions from the preparative isoelectric focusing.

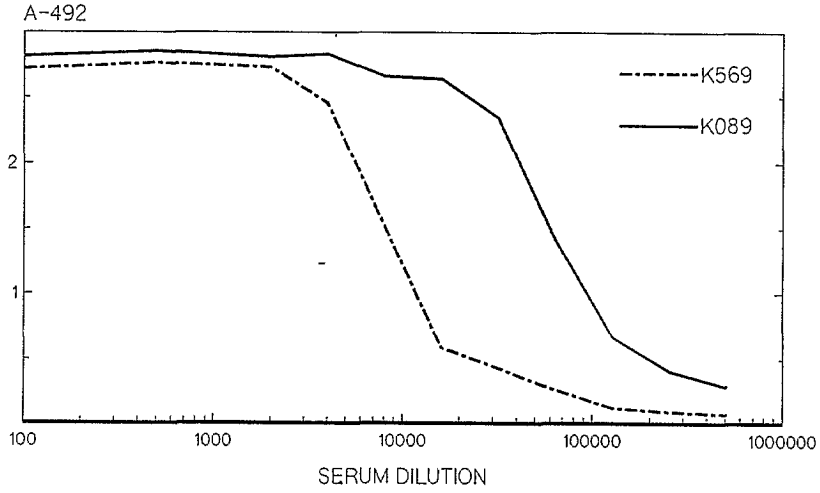

Fig. 3. Sensitivity of antiserum from two rabbits as revealed by ELISA with purified myrosinase. Specific reactivity was obtained even at serum dilutions of 1: 500 000.

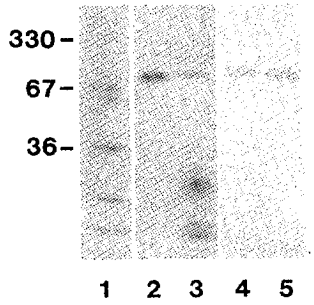

Fig. 4. Western blot of myrosinase from Sinapis alba. (1) Size markers, (2) purified myrosinase, (3) dialyzed and centrifuged crude extract, (4-5) Western blot and immunolabelling of myrosinase in crude extract (4) and purified myrosinase (5), respectively.

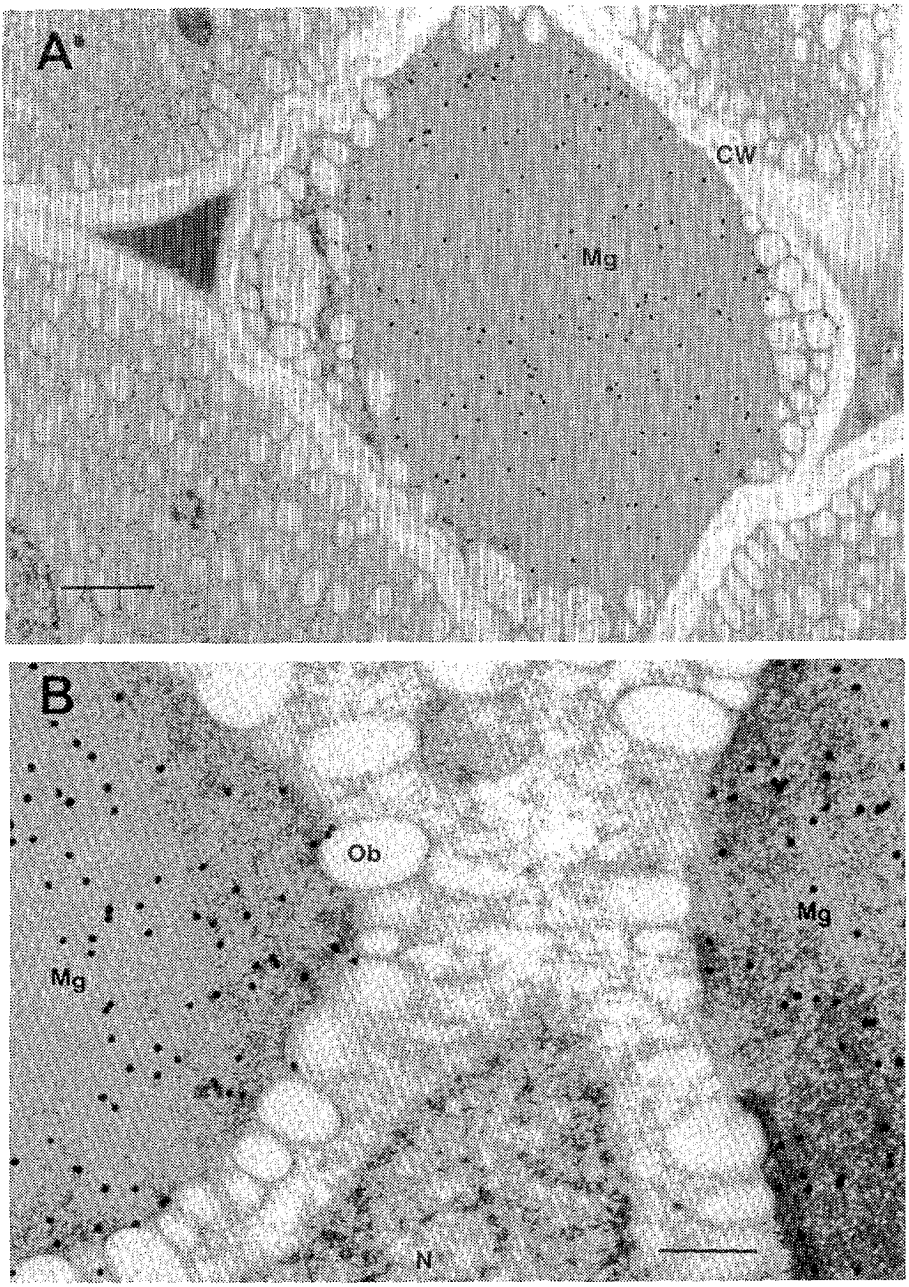

Fig. 5. Immunogold-EM labelling of myrosinase in white mustard radicle (A) and cotyledon (B) 24 h after seeding. Note the specific localization of myrosinase to the myrosin grains. Scale bar A: 1μm, B: 500 nm. Myrosin grain (Mg), cell wall (CW), oil body (Ob), nucleus (N).

ACKNOWLEDGEMENTS

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