MONOCLONAL ANTIBODIES TO MYROSINASE IN PLANTS.

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Abstract:
Myrosinase was extracted and purified from seeds of rape (Brassica napus L.) by ion-exchange chromatography, gel filtration and finally fast protein liquid chromatography (FPLC). Electrophoresis of the highly purified enzyme showed single bands after silver staining both in isoelectric focusing and SDS-PAGE on a gradient gel.
Mice were immunized with partially purified myrosinase, and the titre determined with enzyme linked immunosorbent assay (ELISA). After fusion, cloning and recloning of the hybridoma cells, specificity was tested with highly purified myrosinase. Positive clones were selected and the antibody class determined in two separate immunoassays. Although most of the hybridoma cultures produced IgM antibodies, some IgG producing cultures were also detected. Screening experiments to test the inhibition effect of the monoclonal antibodies on the myrosinase activity gave one positive clone. The myrosinase activity was inhibited by approximately 50%.

Key words:
Hybridoma, monoclonal antibodies, beta-thioglucosidase (myrosinase), Brassica napus L.

Introduction:

Myrosinase (beta-thioglucosidase, E.C.3.2.3.1) catalyses the hydrolysis of glucosinolates, a group of sulphur-containing glycosides present in all Brassicaceae species examined. The glucosinolates are not deleterious

themselves, but the cleavage products isothiocyanates, nitriles or thiocyanates (depending upon substrate and pH of the hydrolysis) can have undesirable effects in animal feedstuff owing to their pungency and goitrogenic activity (Wilkinson et al., 1984). At present, little is known about the localization of myrosinase in plants. Information on myrosinase and its histological localization has been sumarized by Phelan and Vaughan (1980) and Bones and Iversen (1985). However, investigation of the localization of myrosinase has been severely hampered by the lack of cytochemical markers.

Monoclonal antibody technology allows the generation of specific antibodies starting from impure preparations (Köhler and Milstein, 1975) and has proven to be a valuable and powerful tool in studies of structure, function and localization of natural substances. We describe here the generation, identification and characterization of monoclonal antibodies to myrosinase present in rape. In addition to use as cytochemical markers for localization, molecular characterization and fractionation, these monoclonal antibodies may provide the basis for new selection tools in plant cell and gene manipulations. This is the first report of monoclonal antibodies specific for myrosinase to be presented.

Materials and methods:

Enzyme preparation:

Seeds of *Brassica napus* L. cv. Niklas (rape) were finely ground and the myrosinase extracted with buffer A (0.01 M imidazole-HCl buffer, pH 9.0). After dialysis against buffer B (0.01 M imidazole-HCl buffer, pH 6.0), the different forms of myrosinase were separated by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52). The column (2.5 x 15 cm) was equilibrated with buffer B. The adsorbed proteins were displaced by a linear ionic strength gradient, made from 1 l 0.01 M and 1 l 0.1 M imidazole-HCl buffer (buffer C, pH 6.0). After dilution with distilled water, fractions with highest myrosinase activity were run on a DE-52 column (2.5 x 10 cm) using the same gradient. The main myrosinase peak from the ion-exchange chromatography was concentrated in an ultrafiltration cell with a PM-10 (Amicon) membrane to 15

ml and applied to a coloumn of Sephacryl 200 (2.5 x 70 cm) equilibrated with buffer B. The final myrosinase purification was performed with the FPLC-system on a pre-packed mono Q HR 5/5 column (Pharmacia) equilibrated with buffer B and eluted with buffer C. Fraction size was 1.5 ml and flow-rate 1 $ml \cdot min^{-1}$. Rechromatography of the three active fractions obtained, was done after dilution of the samples on the same column, but with a less steep gradient. Isoelectric focusing and SDS-Page were carried out with the PhastSystem (Pharmacia) on PhastGel IEF 4-6.5 and gradient 10-15, respectively. Protein was silver stained.

Monoclonal antibody production:
Balb/c mice (6-8 weeks old) were immunized at day 1, 17, 41, 91 and 92 with 20-100 μg myrosinase preparation obtained after gel filtration on Sephacryl 200. The first immunization was given in presence of 50% Freund's complete adjuvant (Difco). Three days after the last immunization, the spleen of one mouse was taken out and the spleen cells were fused with NSO plasmacytoma cells as described by Eshhar (1985). Following fusion the cell suspension was plated into 484 microwells (Costar) and grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and 100 μM hypoxanthine, 16μM thymidine, 0.4μM aminopterin (HAT). After fourteen days clones of hybridomas could be observed in all wells. Hybridoma supernatants were removed and screened for antibodies against myrosinase in an ELISA assay as described below. The primary screening gave 24 positive hybridomas and fifteen were selected for further analysis. The hybridomas which gave the highest reading in the ELISA assay were cloned by limiting dilution.

Enzyme linked immunosorbent assay of antibody binding:
After each of the following incubation steps, plates were washed three times with PBS supplied with 0.05% Tween (PBS-T). All steps were performed at 37°C and for 30 min if nothing else is stated. ELISA-grade, flat bottom plates (96 wells/plate) were incubated over night at 4°C with 100 μl/well of the myrosinase preparation diluted to 4-6$\mu g \cdot ml^{-1}$ with PBS, washed and blocked with 0.5% bovine serum albumin (RIA-grade, Sigma) in PBS. Each well was incubated

with 100µl of each serum dilution or supernatant, followe by incubation with 50µl of biotinylated antigen specific antibody (Biotin-F(ab')2 fragment of rabbit x mouse Ig(G+A+M+H+L) diluted 1:2000 with PBS (Zymed). Plates were thereafter incubated with 50µl streptavidin-biotin in PBS (SABC-solution, Zymed) at room temperature for 30 min, washed and 100µl substrate solution consisting of 2.8 ml 0.1 M citric acid, 2.2 ml 0.2 M Na_2HPO_4, 5.0 ml dist. H_2O, 10.0 mg ABTS (2,2-azinodi (3-ethylbenzthiazoline sulfonic acid)) and 5µl concentrated H_2O_2, added. The reaction was stopped by adding 100 µl 0.2 M citric acid to each well and results recorded using an automatic microelisa reader at 410 nm.

Characterization of monoclonal antibodies:
The monoclonal class was determined with a monoAb-ID EIA kit from Zymed, as descibed in their general procedure or by the capture method. In both cases visualization was performed with the alkaline phosphatase system.

Myrosinase assay:
Myrosinase activity was tested using GOD-Perid-reagent to determine the amount of glucose liberated from hydrolysis of sinigrin (2-propenylglucosinolate). GOD-Perid contains buffer, peroxidase, glucose oxidase and a chromogen. The absorbance av 420 nm was measured in a spectrophotometer against a blank solution prepared by mixing one samle without enzyme and one without sinigrin, after the boiling step (see e.g Iversen et al., 1979).

Inhibition of myrosinase activity:
Inhibition of myrosinase activity was determined by adding 25 or 50µl hybridoma supernatant to a myrosinase test solution consisting of 29 µl 0.05 M citrate buffer (pH 5.5), 15µl sinigrin (15 $mg \cdot ml^{-1}$), 10 µl myrosinase solution and 140µl GOD-Perid-reagent. Monoclonal antibodies from cell culture supernatants for the above analysis, were used after thorough dialysis against PBS to remove glucose which will interfere in the myrosinase assay based upon the glucose reagent.

Test of spesificity:
Highly purified myrosinase derived from two repetitive runs on FPLC was used to determine the specificity of the generated monoclonal antibodies. Controls were run with fractions from the separation without myrosinase activity.

Results and discussion:

Balb/c mice were immunized with the myrosinase preparation from molecular sieve chromatography on Sephacryl 200, following the schedule previously described by Eshhar (1985) leading to the production of antisera in all mice used. Sera obtained 10 days after the second immunization showed high binding activity against myrosinase in the ELISA assay (Fig.1). This indicates that myrosinase is a highly antigenic protein.

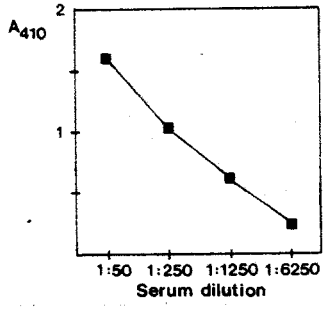
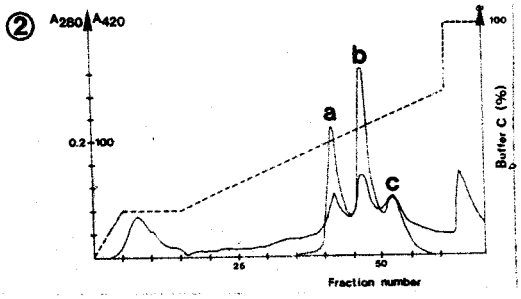


Fig. 1: Titre of serum from the mice used for fusion.



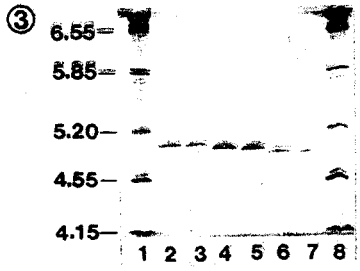


Fig. 2: Separation of the three forms (a, b and c) of myrosinase by FPLC. Ionic strength gradient: 0.01 M - 0.1 M imidazole-HCl buffer (pH 6.0) ----, myrosinase activity (A420)....., protein concentration ——.

Fig.3: Isolectric focusing on a PhastGel IEF 4-6.5 of the three myrosinase forms obtained after the FPLC separation. Pharmacia low pI calibration kit (1 and 8), myrosionase a (2 and 3), myrosinase b (4 and 5) and myrosinase c (6 and 7).

Results from the FPLC separation (Fig.2) and the PhastSystem separation (Fig.3) (Bones, unpublished results) confirmed that this preparation consisted of three myrosinase forms with slightly different isoelectric points (4.94, 4.96 and 5.00), probably the same myrosinase forms as descibed by Lönnerdal and Janson (1973).

From the positive cultures, 15 with high score (Tab.1) in the enzyme linked immunosorbent assay used, were chosen for further expansion. Only hybridoma cultures giving supernatants that exhibited a test-to-control ratio greater than forty in the ELISA were chosen for cloning (M4, M11, M14 and M15, Tab.1). Following cloning by limiting dilution, more than twenty cell lines were obtained that stably secreted antibody.

Culture	C	M1	M2	M3	M4	M5	M6	M7
OD	0.027	0.723	0.956	1.879	1.339	0.388	0.943	0.960

Culture	M8	M9	M10	M11	M12	M13	M14	M15
OD	0.240	0.939	0.547	1.472	0.513	0.473	1.776	1.213

Tab.1: ELISA readings from screening of hybridoma cultures. C: control value.

Isotyping

Knowledge of antibody class and subclass is a great help in determining the strategy of purification and as an indication of monoclonality, and can be determined by several methods (see e.g Zola and Brooks, 1982. Using a set of antisera available commercially from Zymed, most of the cloned hybridoma cultures were found to produce IgM antibodies. But, as shown in Tab. 2, some clones produced IgG2b antibodies. The selection system used, where only hybridoma cultures with high score in the ELISA test were chosen, could account for the high number of IgM secreting hybridoma obtained.

Tab.2: Characterization of selected monoclonal antibodies to myrosinase from rape. Antibody class (isotype), and reactivity against the three froms of myrosinase obtained after the fast protein liquid chromatography (a,b and c), expressed as mutual binding capasity.

Clone	Isotype	Reactivity with FPLC fraction		
		a	b	c
M4- 2C	IgM	+++	+	++
M11-1D	IgM	+++	+++	+++
M14-5A	IgM	+++	++	+++
M15-2G	IgG2b	+	+	+

Myrosinase inhibition:
Screening experiments to test the inhibition effect of the monoclonal antibodies on myrosinase activity, gave one positive clone (M15-2G). As showed in Fig. 3, this antibody decreased the myrosinase activity with 50%. This result indicates that the M15 antibody binds to a site associated with the function of the myrosinase.

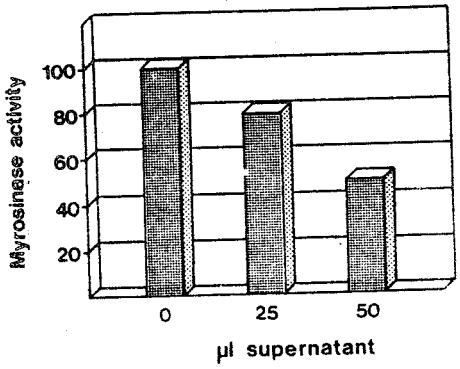


Fig.3: Inhibition of myrosinase activity by monoclonal antibodies from clone M15-2G. The values are given as per cent of the activity in the control samples, which contained the same amounts of supernatant as the test samples, but from a non-inhibiting clone.

References:

Bones, A. and T.-H. Iversen, 1985. Myrosin cells and myrosinase. Israel Journal of Botany, 34: 351-376.

Eshhar, Z., 1985. Monoclonal antibody strategy and techniques. In: Springer, T., ed. Hybridoma technology in the biosciences and medicine. Plenum Press, New York, pp. 3-41. ISBN 0-306-41996-3.

Iversen, T.-H., C. Baggerud and T. Beisvåg, 1979. Myrosin cells in Brassicaceae roots. Zeitschrift für Pflanzenphysiologie, 94: 143-154.

Köhler, G. and C. Milstein, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 256: 495-497.

Lönnerdal, B. and J.-C. Janson, 1973. Studies on myrosinase. II. Purification and characterization of a myrosinase from rapeseed (Brassica napus L.). Biochimica et Biophysica Acta. 315: 421-429.

Phelan, J.R. and J.G. Vaughan, 1980. Myrosinase in Sinapis alba L. Journal of Experimental Botany, 31: 1425-1433.

Wilkinson, A.P., M.J.C. Rhodes and R.G. Fenwick, 1984. Myrosinase activity in Cruciferous vegetables. Journal of the Science of Food and Agriculture, 35: 543-552.

Zola, H. and D. Brooks, 1982. Techniques for the production and characterization of monoclonal hybridoma antibodies. In: Hurrel, J.G.R., ed. Monoclonal hybridoma antibodies. Techniques and application. CRC Press. ISBN 0-8493-6511-2.