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FAST POLYMER LIQUID CHROMATOGRAPHY ISOLATION AND CHARACTERIZATION OF PLANT MYROSINASE, β-THIOGLUCOSIDE GLUCOHYDROLASE, ISOENZYMES

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SUMMARY

Rapid and high-yielding ion-exchange chromatographic and chromatofocusing methods using fast polymer liquid chromatography (FPLC) have been developed for plant myrosinase, β-thioglucoside glucohydrolase (EC 3.2.3.1), isoenzyme studies. The preparative FPLC methods showed a mean recovery of 85% for six myrosinase preparations from three different plant sources. FPLC chromatofocusing and analytical isoelectric focusing were used to demonstrate the presence of different mixtures of myrosinase isoenzymes in seeds of various crucifers. The stability of the isoenzymes was investigated. The proposed method of plant isoenzyme analysis is briefly discussed in relation to methods previously used for myrosinase isolation and characterization, and studies of glucosinolate catabolism.

INTRODUCTION

β-Thioglucoside glucohydrolase (EC 3.2.3.1) (myrosinases) occur in all glucosinolate-containing plants hitherto investigated[1]. Myrosinases catalyse the hydrolysis of glucosinolates (Fig. 1), thereby producing D-glucose and the unstable thiohydroxamate-O-sulphonates, which are further degraded to sulphate and various amounts of different products[1]. Only limited information on glucosinolate catabolism *in vivo*, including the possible role of myrosinases, is available[1,2]. It is, however, well established that both the intact glucosinolates and their degradation products are important quality factors for oilseed rape[3–5] and for other glucosinolate-containing plants used as food and feed[6]. Knowledge of myrosinases and glucosinolates is also important in relation to studies of the interactions between herbivores and glucosinolate-containing plants[7,8].

Myrosinases in crucifers are glycoproteins containing several thiol and disulphide groups, and they often exist as isoenzymes[9–15]. The reports concerning isoenzymes are not always in agreement with each other. Pihakaski and Pihakaski found

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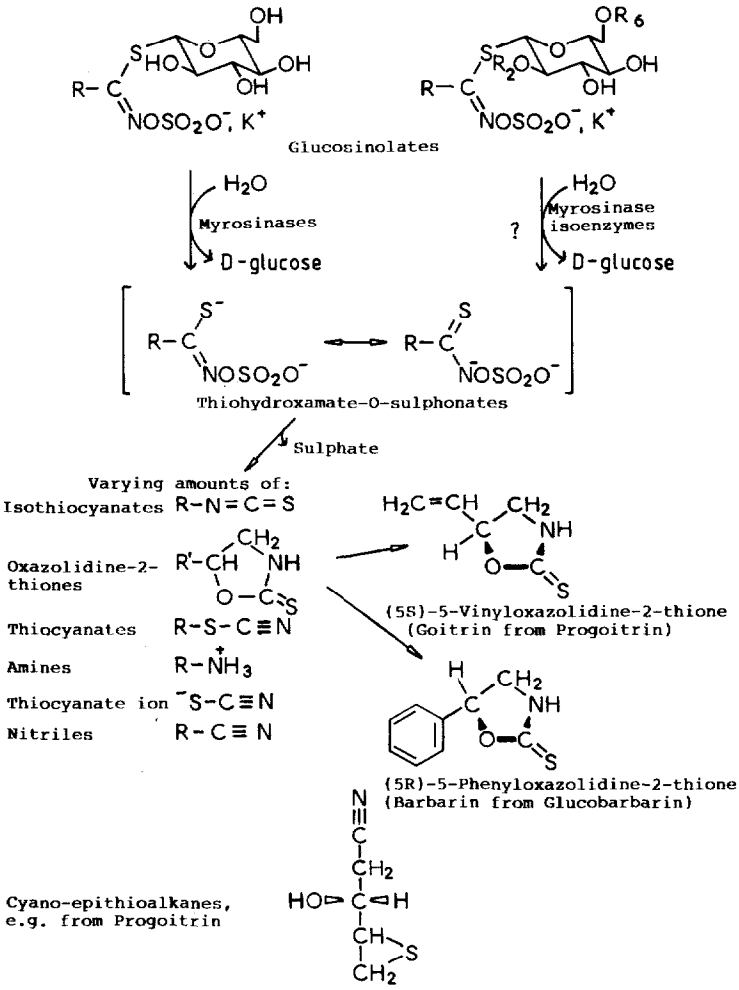

Fig. 1. Names and structures of degradation products of glucosinolates formed by myrosinase-catalysed hydrolysis: R = side-chain of glucosinolates; R_2 and/or R_6 = H or cinnamoyl derivatives. Glucosinolates are constituents of crucifers, but information on myrosinases attacking glucosinolates substituted with cinnamoyl derivatives is not yet available.

that the pattern of isoenzymes in *Sinapis alba* changed during germination[16], whereas Phelan and Vaughan found no differences in the isoenzyme pattern in seeds and seedlings of *S. alba*[17].

The isoenzyme pattern does not always seem to be stable. It was found to depend on the method of enzyme preparation[13], and the results should therefore be treated with caution. Some of the enzyme bands could originate from dissociation of enzyme aggregates or other alterations of the original enzyme molecule[13], or the enzyme could be composed of subunits that could associate and dissociate[13,15].

In seeds of *S. alba*, one glucosinolate, *p*-hydroxybenzylglucosinolate, constitutes *ca.* 95% of the total glucosinolate content, but as many as fourteen myrosinase

isoenzymes have been detected[17]. The reason for the presence of isoenzymes is not yet understood. It is, however, important to stress that most of the studies concerning myrosinase isoenzymes hitherto performed have been based on methods with low recoveries[9–21]. This is clearly a serious drawback with respect to quantitative behaviour and probably a cause of the inconsistencies in the literature[19–21].

This paper presents a new, rapid and high-yielding fast polymer liquid chromatographic (FPLC) technique for the isolation and characterization of plant myrosinase isoenzymes. The method is promising with respect to isoenzyme studies, *e.g.* in plant-breeding programmes or in clarification of the hitherto ambiguous results. It is the first report of a purification and stability study of the plant myrosinase isoenzymes by means of FPLC.

EXPERIMENTAL

Myrosinase assay

After mixing with enzyme solution, the decrease in A_{233} for a 33 μM sinigrin (allylglucosinolate) solution in McIlvaine buffer (pH 6.70) was measured (a modification of the method described by Schwimmer[22]). One unit (U) was defined as the amount of enzyme activity that hydrolysed 1 μmol of sinigrin per minute at 25°C and pH 6.70.

Protein determinations

The Coomassie brilliant blue method[23] was applied to crude extracts and ethanol powder preparations, using only 5 ml of reagent and bovine serum albumin as standard. For the fractions from FPLC, A_{280} and A_{260} were measured, and a correction for the content of nucleic acids was performed according to Warburg and Christian[24].

Spot test for myrosinase activity

This test was performed by mixing 50–100 μl of sinigrin solution (1 mg of sinigrin, 5 mg of barium chloride, 0.5 mg of ascorbic acid and 0.1 ml of acetic acid per millilitre of solution) and an equal volume of fractions from FPLC. The presence of myrosinase activity was visualized as a precipitate of barium sulphate.

Crude extracts

Seeds of *Brassica nigra* (L.) Koch, *B. napus* L. and *S. alba* L. were ground and extracted with water (45 ml/10 g meal) by use of an Ultra Turrax homogenizer (3 × 30 s, 2°C). Magnetic stirring (1 h, 5°C) yielded a suspension which, after centrifugation (14 000 *g*, 15 min, 4°C) and filtration through cheese-cloth, resulted in a crude extract.

Ethanol powder

Ethanol (96%, −15°C) was added dropwise to the crude extract (1:1). Centrifugation as above yielded a pellet, which was washed with ethanol (70%, 8 vols.). Centrifugation as above yielded a pellet, which was dissolved in water by stirring (50 ml, 10 min, 5°C). Centrifugation was performed again as above. The lyophilized supernatant was the ethanol powder.

Isolation of myrosinases by FPLC

Instrumentation. This consisted of Pharmacia gradient programmer GP-250, Pharmacia chromatography rack, Pharmacia single path monitor UV-1 (280 nm), fraction collector FRAC-100, Pharmacia two-channel recorder REC 482, two pharmacia high precision pumps P-500 and a valve V-7.

FPLC ion-exchange chromatography. A pre-packed mono Q HR 5/5 column (Pharmacia) equilibrated with buffer A (20 m*M* Tris, pH 7.50) was used. Elution gradient, 0–100% buffer B (20 m*M* Tris, 1 *M* sodium chloride, pH 7.50) in 10 min, followed by 100% B for 2 min; flow-rate, 1 ml/min; fraction size, 1 ml. To isolate myrosinase from *B. nigra*, the crude extract (500 μl) was applied for the first ion-exchange step, followed by re-chromatography of the obtained myrosinase-containing fraction (500 μl). To perform ion-exchange chromatography of ethanol powder preparations from the three species, ethanol powder (50 mg) was dissolved in buffer A (2.5 ml) and 1.3 ml were chromatographed in three consecutive runs.

FPLC chromatofocusing. A prepacked Mono P HR 5/20 column (Pharmacia) was used. It was equilibrated with buffer A (25 m*M* bis-Tris, pH 7.10). The column was eluted with 2 ml of buffer A, followed by 46 ml of buffer B [Polybuffer 74 (Pharmacia) diluted with water (1:9, pH 4.00)] and 2 ml of buffer A. The flow-rate and fraction size were as above. For the three species, 1 ml of the purest fraction from the ion-exchange step was applied to the column after changing the solvent to buffer A on a Sephadex G-25 column. The pH in the fractions was measured with a glass electrode (Radiometer). For *S. alba* myrosinase, re-chromatofocusing was performed on four fractions from the first separation.

Isoelectric focusing (IEF)

Agarose gel IEF. This was performed as described in the manufacturer's instructions (Pharmacia). Ampholine (LKB) pH 3.5–10 was used. A crude extract (20 μl) of *S. alba* was applied on paper (0.5 × 1 cm). Electrophoresis was carried out for 2.5 h, at 500 V, 15–20 mA, and 8°C. To determine the pH gradient, gel pieces (1 × 1 cm) were extracted with water (5 ml, 1 h, 20°C), and the pH in the solutions was measured. Myrosinase activity bands were developed as described for the spot test (a modification of the method by MacGibbon and Allison[10]). The gel was pressed and dried after washing with ethanol–acetic acid–water (35:10:55) (2 × 15 min).

Polyacrylamide gel IEF. LKB Ampholine PAG plates 1804-101, pH 3.5–9.5 were used as described in the manufacturer's instructions. Ethanol powder solution (50 μl) from the three species, as well as fractions from the ion-exchange and chromatofocusing steps, were applied by soaking paper strips in the solutions. Electrophoresis was carried out for 16 h, at 250 V, 4 mA, and 8°C. Gels were stained as above.

RESULTS

Fig. 2 shows chromatograms from the isolation of myrosinases from *B. nigra* crude extract by a two-step FPLC ion-exchange chromatography procedure, resulting in a 26-fold purification.

Yields and purifications from FPLC ion-exchange chromatography and chromatofocusing of myrosinase isoenzymes from ethanol powders of the three examined species are shown in Table I: the mean recovery is 85%.

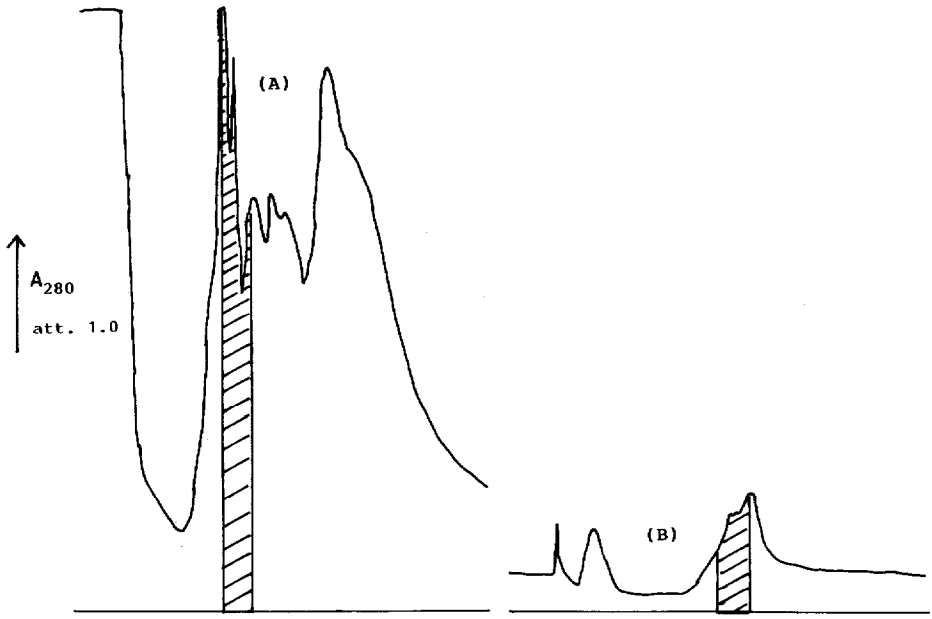

Fig. 2. FPLC ion-exchange chromatography of myrosinase crude extract from *B. nigra*: (A) first ion-exchange step; (B) re-chromatography of fraction 7.

FPLC chromatofocusing analysis of myrosinases from the examined species resulted in the following p*I* intervals for the enzymes: *B. nigra*, pH 5.14–5.33; *B. napus*, pH 5.61–5.90 and *S. alba*, pH 5.63–6.15. Fig. 3 shows an elution profile (pH) and the fractions containing myrosinase activity (three runs).

Results from the complete isolation procedure for myrosinase isoenzymes from seeds of *S. alba* appear in Table II, together with results from re-chromatofocusing of some of the fractions.

IEF investigations of the isoenzymes from *B. nigra*, *B. napus* and *S. alba* are shown in Figs. 4–6.

TABLE I

PURIFICATION OF MYROSINASE ISOENZYMES FROM SEEDS OF CRUCIFERS

Species	*FPLC ion-exchange chromatography of ethanol powder*		*FPLC chromatogocusing of ion-exchange fractions*	
	Recovery (%)	*Purification*	*Recovery (%)*	*Purification*
Brassica nigra	80	3.6× (Fr. 7)*	91	2.8×
Brassica napus	76	4.1× (Fr. 7)	71	2.5×
Sinapis alba	84	1.7× (Fr. 6)	114	5.3×

* Fr. 7 = fraction 7, etc.

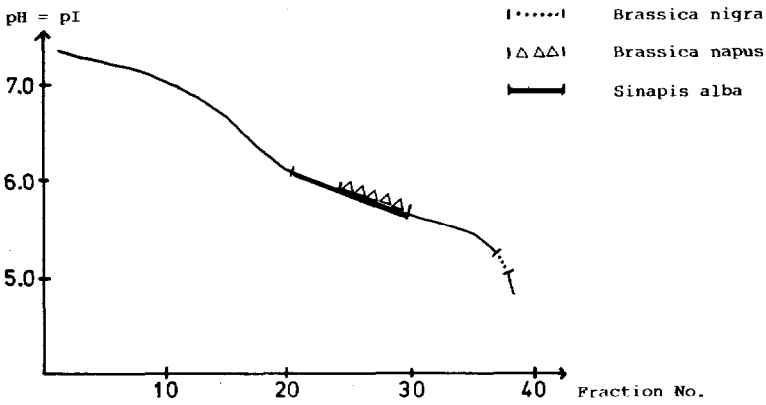

Fig. 3. Elution curve (pH) obtained by FPLC chromatofocusing of myrosinase isoenzymes from seeds of crucifers.

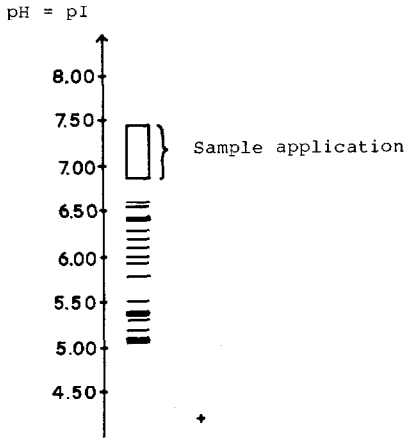

Fig. 4. Agarose IEF of crude extract from *S. alba*. The gel was stained for myrosinase activity.

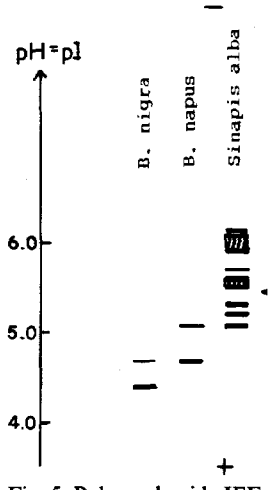

Fig. 5. Polyacrylamide IEF of ethanol-powder preparations. The gel was stained for myrosinase activity.

TABLE II

ISOLATION OF MYROSINASE ISOENZYMES IN *S. ALBA* BY FPLC

	Protein (mg/ml)	*Activity (U/ml)*	*Total activity (U)*	*Specific activity (U/mg protein)*	*Purification*	*pI*
Crude extract (200 ml)	28.1	0.3755	75.1	0.0134		
Ion-exchange chromatography						
Ethanol powder*	2.40	2.8493	3.7041	1.19	89×	
Fr. 2**	1.864	0.2549		0.14		
Fr. 3	0.836	0.2174		0.26		
Fr. 4	0.034	0.0319	3.1185	0.94		
Fr. 5	0.015	0.0041	(84%)	0.27		
Fr. 6	0.196	0.4047		2.06	154×	
Fr. 7	0.174	0.1265		0.73		
Chromatofocusing						
Fr. 6***	0.099	0.1537	0.1537	1.55		
Fr. 20	0.012	0.0032		0.27		6.15
Fr. 21	0.001	0.0083		8.30	619×	6.08
Fr. 22	0.021	0.0340		1.62	121×	6.03
Fr. 23	0.023	0.0184		0.80		5.97
Fr. 24	0.042	0.0286	0.1758	0.68		5.91
Fr. 25	0.019	0.0368	(114%)	1.94	145×	5.85
Fr. 26	0.010	0.0214		2.14	160×	5.80
Fr. 27	0.014	0.0189		1.35		5.74
Fr. 28	0.024	0.0037		0.15		5.68
Fr. 29	0.011	0.0025		0.23		5.63
Re-chromatofocusing						
Fr. 21 + 22, 0.5 ml see above		0.0313	0.0157			
Fr. 21, 1.0 ml		0.0042	0.0042 (27%)			5.70
Fr. 27 + 28, 0.5 ml see above		0.0140	0.0070			
Fr. 25, 1.0 ml		0.0023	0.0023 (33%)			5.40

* Ethanol powder (50 mg) was dissolved in 2.5 ml of buffer A, and 1.3 ml of this enzyme solution were chromatographed by three identical runs. Each fraction consisted of 1 ml.

** Fr. 2 = fraction 2, etc.

*** Fr. 6 was diluted by changing the buffer in a G-25 column before it was applied to the column. Each fraction consisted of 1.0 ml.

DISCUSSION

Some of the previously reported investigations on myrosinase isoenzymes using traditional methods of analysis suffered from low recoveries, which probably explain the inconsistent results[11,16–21].

No reports of the FPLC technique now used for myrosinase separation have appeared previously. As shown by the results in Fig. 2 and Tables I and II, excellent

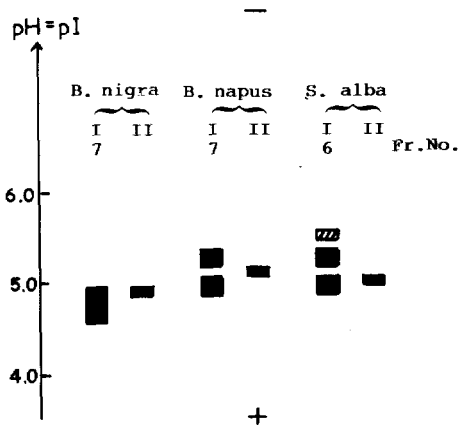

Fig. 6. Polyacrylamide IEF of the ion-exchange preparations used for chromatofocusing (I) and the purest preparation obtained by this purification (II). The gel was stained for myrosinase activity.

recovery and purification of myrosinase can be obtained by this FPLC technique. The high yields of the FPLC techniques almost certainly results from the rapidity of the methods, which ensures that the enzymes are exposed to potentially damaging conditions for only a very short time. Previously, the FPLC technique has successfully been used for isolation of other plant enzymes[25].

The myrosinase isoenzyme bands visualized in Fig. 4 indicate that myrosinase in *S. alba* crude extract consists of at least fourteen isoenzymes[17]. In the ethanol-powder preparation of the crude extract, this number is reduced, (Fig. 5), indicating that myrosinase preparations should be treated very carefully during isolation and characterization of the enzymes, especially when comparative studies of isoenzyme patterns are performed. This is in agreement with previously reported results[10,13,14].

Results from the chromatofocusing steps presented in Fig. 3 show that the three cruciferous species contain different myrosinase isoenzymes. The relative p*I* values for the isoenzymes are confirmed by the IEF results in Figs. 5 and 6. The p*I* values obtained in the chromatofocusing method differ from those obtained in the IEF experiments. This is caused by the differences between these two techniques, and only the relative positions of the isoenzymes should be compared. IEF of the ethanol-powder preparations (Fig. 5) shows that myrosinase isoenzymes from *B. nigra* are characterized by rather low pI values compared with myrosinase isoenzymes from *S. alba*. The isoenzymes from *B. napus* have p*I* values between those of the two other species. The same pattern is found in the IEF investigations of the ion-exchange preparations used for chromatofocusing (Fig. 6).

After ion-exchange chromatography, myrosinase activity is found in various amounts depending on the species in fractions 2–7; the majority containing the highest specific activity appear in fractions 6 and 7. The numbers of bands containing enzymatic activity by IEF before (Fig. 5) and after (Fig. 6) the FPLC ion-exchange chromatography step are similar, although the *S. alba* pattern spans less than one pH-unit after the step. This indicates that isoenzymes with higher p*I* values are not found in the later fractions of the ion-exchange step. The number of bands containing

enzymatic activity by IEF before and after the FPLC chromatofocusing step are not comparable in Fig. 6, because only the purest preparation of this step (containing one band) is shown. Other experiments (not shown) have shown that the myrosinase isoenzymes are well separated according to their p*I* values by chromatofocusing, the isoenzymes with the higher p*I* values being eluted first from the column.

In order to investigate the stability of the isoenzymes in fractions obtained from FPLC chromatofocusing, re-chromatofocusing was carried out on *S. alba* isoenzymes. The results show (Table II) that by re-chromatofocusing of the fractions (21 and 22) and fractions (27 and 28), the relative p*I* values are reproduced. The mean difference between the p*I* values of the two samples is 0.35 pH units in the first run compared with 0.30 pH unit in the re-chromatofocusings. The reproducibility shows the stable nature of the isoenzymes. If the enzymes in *S. alba* were subjected to a continuous turn-over, re-chromatofocusing of a fraction with a specific p*I* value would be expected to yield enzymes spread over a broad p*I* range.

The re-chromatofocusing and the IEF investigations indicate that myrosinases in *B. nigra*, *B. napus* and *S. alba* are composed of a mixture of isoenzymes with rather reproducible p*I* values. The three plant species each show a specific individual isoenzyme pattern.

The results presented here show that FPLC can be used successfully for a more detailed study of myrosinase isoenzymes. The rapidity and the high recovery imply that FPLC could be used to isolate the isoenzymes in a screening of myrosinases from different crucifers, for plant-breeding programmes, and for studies of glucosinolate catabolism and catalytical function–mechanism relationships of the different isoenzymes.

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