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RAPID AND EFFICIENT METHOD FOR THE ISOLATION AND CHARACTERIZATION OF PLANT AROMATIC CHOLINE ESTERASES

LONE M. LARSEN, TOM H. NIELSEN, ANNETTE PLÖGER and HILMER SØRENSEN*
Chemistry Department, Royal Veterinary and Agricultural University, 40, Thorvaldsensvej, DK-1871 Frederiksberg C (Denmark)

SUMMARY

Aromatic choline esterases have been isolated from seedlings of six crucifers. A fast polymer/protein liquid chromatographic (FPLC) method was developed for the final purification and characterization of the enzymes. FPLC anion-exchange chromatography and chromatofocusing resulted in highly purified enzymes. The elution profiles of the enzymes from different plant species showed appreciable differences. Chromatofocusing revealed differences in their relative p*I* values (4.3–5.1) and allowed the separation of aromatic choline esterase isoenzymes. Chromatofocusing revealed that two isoenzymes of aromatic choline esterases occur in *Barbarea intermedia*. The molecular weight of the enzyme from *Sinapis alba* was calculated to be about 40 000 daltons by gel filtration. Kinetic studies confirmed the existence of different aromatic choline esterases in plants.

INTRODUCTION

Aromatic choline esters are a group of natural products widespread in glucosinolate-containing plants (*e.g.*, crucifers)[1–7] and orchidaceae[8]. Often they accumulate appreciably in the seeds, sinapine being the best known example in *Brassica*[9]. Several other types of aromatic choline esters can be quantitatively dominant among the aromatic choline esters[9]. Examples of dominating aromatic choline esters in other plants are *p*-hydroxybenzoylcholine in *Sinapis*[7], sinapine glucoside in *Alliaria*[5], isoferuloylcholine[3] in *Barbarea*[10] and a lignan type in *Brassica nigra*[10].

During germination of the seeds, the aromatic choline esters are catabolized[7,11]. The initial step is the hydrolysis of the esters, which is catalysed by aromatic choline esterases[11]. Knowledge about the enzymes is sparse and based almost solely on sinapine esterase isolated from seedlings of *Brassica hirta* Moench.[12] and *Raphanus sativus* L.[13]. Additional information about aromatic choline esterases is therefore needed, especially owing to the increasing interest in aromatic choline esters and products thereof in relation to the quality of oilseed rape[9,11].

In this work, aromatic choline esterases were isolated from the seedlings of six crucifers. The properties of highly purified enzymes were investigated and compared

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with previously reported data for sinapine esterase. A combination of fast polymer/protein liquid chromatographic (FPLC) techniques has been developed for the purpose of obtaining rapid information about the status of aromatic choline esterases in various crops. The application of FPLC resulted in a rapid and effective method for the study of the presence and properties of these enzymes, including the separation of isoenzymes. Further, a comparison of the kinetic parameters for the enzymes, including five different substrates found in varying amounts in the seeds of the six plants, was performed for a more detailed characterization of the differences between the enzymes.

EXPERIMENTAL

Plant material

Seedlings were produced as described elsewhere[11] from *Sinapis alba* L., *Brassica napus* L. cv. Line and cv. Gulliver, *Brassica nigra* (L.) Koch cv. Junius, *Brassica campestris* L. cv. Candle and *Barbarea intermedia* Bor.

Determination of aromatic choline esters

HPLC methods described elsewhere were used[9,14].

Enzyme assay

Sinapine thiocyanate (100 μM) was prepared in McIlvaine buffer (pH 6.50); the total volume was 1545 μl. After addition of enzyme, dA_{350}/dt was measured against buffer. One enzyme unit (U) = 1 μmol of sinapine hydrolysed per minute (25°C), resulting in

$$\text{U/ml enzyme solution} = \frac{1545}{b\,(\varepsilon_{350,\,\text{sinapine}} - \varepsilon_{350,\,\text{sinapic acid}})} \cdot \frac{-dA}{dt} = \frac{1.545}{8b} \cdot \frac{-dA}{dt}$$

where cell path length = 1.00 cm, $\varepsilon_{350,\,\text{sinapine}} = 8900\ \text{l mol}^{-1}\ \text{cm}^{-1}$, $\varepsilon_{350,\,\text{sinapic acid}} = 900\ \text{l mol}^{-1}\ \text{cm}^{-1}$ and b = ml of enzyme solution used in assay.

Protein determination

The Coomassie Brilliant Blue method with bovine serum albumin as a standard was used[15]. Fractions from FPLC were measured at 280 nm.

Enzyme spot test

Acetylthiocholine iodide in McIlvaine buffer (pH 6.50) (75 mM, 5 μl), 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent; Sigma, St. Louis, MO, U.S.A.) in McIlvaine buffer (pH 8.00) (10 mM, 25 μl) and a maximum of 100 μl of enzyme solution were mixed with McIlvaine buffer (pH 8.00) in a total volume of 230 μl[11]. The colours were compared visually after 20 min.

Crude extract

Lyophilized seedlings (10 g) were homogenized in deionized water (150 ml; 4°C) by use of an Ultra Turrax homogenizer (5 × 20 s). Centrifugation for 60 min at 11 000 g (4°C) and filtration through filter-paper yielded about 120 ml of crude extract.

Ammonium sulphate precipitation

Addition of solid ammonium sulphate and magnetic stirring at 4°C for 30 min, followed by centrifugation for 60 min at 11 000 *g* (4°C), yielded a precipitate between 21 and 63% of salt, which was dissolved in 30 ml water and centrifuged for 2 h as above. The supernatant contained the aromatic choline esterases.

Ultrafiltration

For concentration and removal of salt, the supernatant from the ammonium sulphate purification step was diluted to 100 ml with water, followed by reduction of the volume to 30 ml in a PM 30 ultrafiltration unit (Amicon, Danvers, MA, U.S.A.). Water (70 ml) was added and the volume was reduced to 10 ml. The filter was washed with 10 ml of 0.1 *M* sodium chloride solution, followed by 10 ml of water. The wash solutions were added to the 10 ml of concentrated solution and the mixture was lyophilized.

Gel chromatography

The powder from the ultrafiltration was dissolved in 10 ml of water (magnetic stirring for 30 min) and the solution was centrifuged for 1 h at 20 000 *g* (4°C). The supernatant (4–5 ml) was applied to a gel chromatographic column (Sephadex G-75; 100 × 2.6 cm I.D.) (Pharmacia, Uppsala, Sweden), equilibrated with McIlvaine buffer (pH 6.50). This buffer was used for elution (10-ml fractions; flow-rate 60 ml/h). The fractions containing aromatic choline esterase activity were pooled, and the salt content was reduced to 1/2000 by means of the ultrafiltration unit (see above) and addition of water.

Isolation of aromatic choline esterase by FPLC

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

FPLC anion-exchange chromatography. A prepacked column (Mono Q HR 5/5, 5 cm × 5 mm I.D.; Pharmacia) equilibrated with buffer A (20 m*M* Tris, pH 7.60) was used. Buffer B was 20 m*M* Tris–1.0 *M* sodium chloride (pH 7.60). Gradient elution was performed with 2 ml of A; 10 to 35% B in 20 min; 2 ml of B; 2 ml of A; 1 ml/min; 1 ml per fraction. To perform ion-exchange chromatography on the G-75 preparation, the freeze-dried product was dissolved in 4.0–6.0 ml of buffer A and the solution was filtered through a 0.4-μm filter (Millipore, Milford, MA, U.S.A.). Two or three consecutive chromatograms of 1.0-ml samples were obtained.

FPLC chromatofocusing. A prepacked Mono P HR 5/20 column (20 cm × 5 mm I.D.; Pharmacia) was equilibrated with buffer A [25 m*M* bis-Tris, pH 6.30 (adjusted with hydrochloric acid)]. The column was eluted with 3 ml of buffer A followed by 46 ml of buffer B. [Polybuffer 74 (Pharmacia) diluted with water (1:9) (pH 3.70) (adjusted with hydrochloric acid)]. The flow-rate and fraction size were as above. A 200–500-μl volume of the purest fractions from the ion-exchange chromatogram were applied to the column after filtration through a 0.4-μm filter. The pH of the fractions was measured with a glass electrode (Radiometer, Copenhagen, Denmark). After each run, the column was rinsed with 1.0 ml of 2 *M* sodium chloride solution.

Molecular weight

Gel chromatography (Sephadex G-200; Pharmacia) was performed on two columns (30 × 1.5 cm I.D.) combined in series. McIlvaine buffer (pH 6.50) was used to dissolve the sample and for elution (5 ml/h). The reference compounds used were blue dextran (2×10^6 daltons; Pharmacia), and gel filtration standard (Bio-Rad Labs., Richmond, CA, U.S.A.) containing five compounds with molecular weights from 670 000 to 1350 daltons.

Optimum pH

Assay mixtures were as mentioned above, except for the McIlvaine buffers (pH 3.00, 5.00, 5.50, 6.00, 6.50, 7.00, 7.50 and 8.00). At 5-min intervals, 150-μl samples were mixed with 150 μl of 2 *M* perchloric acid and, after centrifugation, 90-μl portions of the supernatant were used for sinapine determination by isocratic HPLC[9]. A correction for non-enzymatic hydrolysis of sinapine at alkaline pH was found not to be necessary.

Gel electrophoresis

Polyacrylamide gel (7%; Tris buffer, pH 8.40) (Bio-Rad Labs.) was used. After electrophoresis, some gels were stained for proteins with Coomassie Brilliant Blue. Other gels were placed for 16 h in a developing solution [70 m*M* maleic acid–170 m*M* sodium sulphate–4 m*M* copper(II)sulphate pentahydrate–20 m*M* glycine–3 m*M* magnesium chloride–4 m*M* acetylthiocholine iodide; pH 6.50]. By this method, which is a modification of the procedure of Brock and Hayward[16], bands containing choline esterase activity became visible as a white precipitate of copper thiocholine.

Kinetics

McIlvaine buffer (pH 6.50) was used throughout the studies. The substrates used are given in Table I. The activity was determined for at least five concentrations in the following ranges: sinapine, 5–105 μM; isoferuloylcholine and *p*-coumaroylcholine, 3–15 μM; and hesperalin and *p*-hydroxybenzoylcholine, 3–25 μM.

Hydrolysis was monitored in a thermostated (25°C) MPS-2000 spectrophotometer (Shimadzu, Kyoto, Japan) by recording dA/dt at a fixed wavelength (see below).

Substrate solution (1500 μl in buffer) was added to a fixed amount of enzyme (30–100 μl) and dA/dt was followed for about 6 min, except for *p*-hydroxybenzoylcholine (40 min), at the wavelengths shown in Table I. v_0 (μmole/min) was obtained from the equation

$$v_0 = (1500 + b)\,(-dA/dt)(1/\varepsilon)$$

where b = μl of enzyme added, v_0 = initial velocity, t = time (min), $\varepsilon = \varepsilon_{\text{choline ester}} - \varepsilon_{\text{acid}}$ and cell path length = 1 cm.

The purified enzymes from the FPLC ion-exchange chromatogram were used for kinetic studies; for *B. napus* cv. Line, the ultrafiltered ammonium sulphate precipitate was used. The crude extract from *S. alba* was used for hydrolysis of *p*-hydroxybenzoylcholine. Interfering coloured substances were eliminated from the *S. alba* crude extract by filtration of 2.50 ml on Sephadex G-25 Medium material in a column (8 × 0.6 cm I.D.). The eluent was water.

TABLE I

SUBSTRATES AND THEIR SOURCES FOR THE KINETIC STUDIES

Measuring wavelengths and corresponding ε values determined in the assay system for substrates and products of the enzyme reactions (pH 6.50).

Substrate and product	*Source*	λ *(nm)**	ε *(l mol^{-1} cm^{-1})*	*Ref.*
Sinapine thiocyanate	*B. campestris*	350	8900	14
Sinapic acid			900	
Isoferuloylcholine acetate	*B. intermedia*	335	14 390	10
Isoferulic acid			3690	
p-Coumaroylcholine acetate	Synthetic	330	13 740	14
p-Coumaric acid			1600	
p-Hydroxybenzoylcholine acetate	Synthetic	270	10 030	14
p-Hydroxybenzoic acid			2330	
Hesperalin acetate	*Hesperis matronalis*	300	5000	10
3,4-Dimethoxybenzoic acid			440	

* λ indicates the wavelength with the greatest difference between substrate and product absorbances.

RESULTS AND DISCUSSION

Determination of enzyme activity at different pH values revealed no distinct optimum, but appreciable activities were found at pH 6–8. Linear relationships between enzyme concentrations and rates of hydrolysis were also found.

Optimal utilization of fast chromatographic techniques calls for a fast spot test. The intensity of the yellow colour in the spot test corresponds to the activity measured in the sinapine assay, except for the aromatic choline esterases from *Barbarea intermedia*. Apparently, the latter enzymes showed a much lower activity for the substrate acetylthiocholine than the other choline esterases. Activity was also revealed by gel electrophoresis.

Fig. 1 shows chromatograms from the development of the FPLC anion-exchange chromatographic techniques used for the purification and characterization of plant aromatic choline esterases. A change in the sodium chloride concentration gradient in the elution buffer (from a to c, Fig. 1) resulted in an efficient separation of the aromatic choline esterase from other seed proteins. The resulting method showed excellent reproducibility, *i.e.*, the esterase activity of a given plant species always appeared at the same sodium chloride concentration in the gradient, the sodium chloride concentration being different for the six species examined (Table II).

In some instances, the flat gradient resulted in a lower recovery of the enzyme than that obtained by use of a gradient from 0 to 1.00 *M* sodium chloride in 20 min. As a compromise between recovery, purification and the need to compare the different enzyme sources, chromatographed under the same conditions, a gradient from 0.10 to 0.35 *M* sodium chloride was used for ion-exchange chromatography. In order to overcome the capacity limitations of the column (total 25 mg of protein) and of the

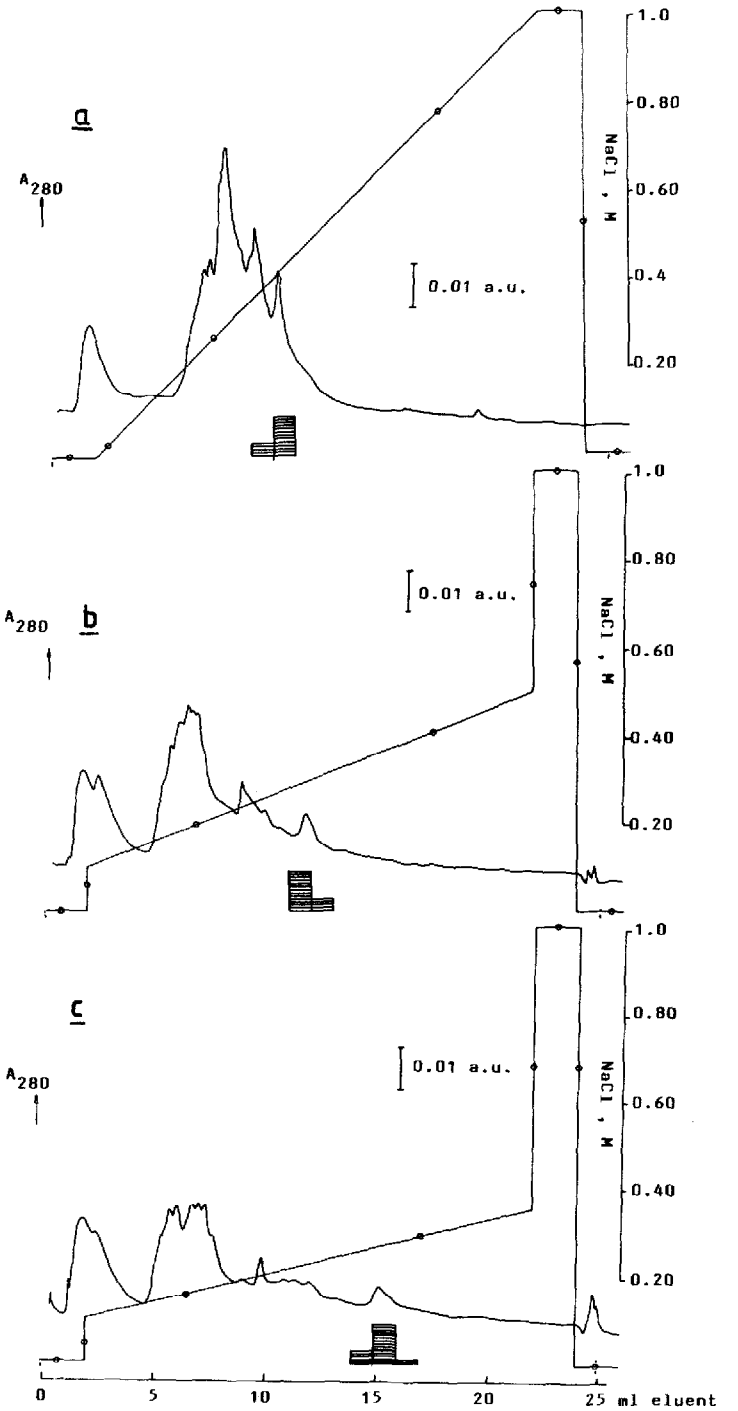

Fig. 1. Optimization of the FPLC ion-exchange isolation of aromatic choline esterase from *Brassica napus* cv. Gulliver. ——, A_{280}; ○—○, concentration of NaCl in eluent. The fractions containing enzyme activity are shaded.

TABLE II

SODIUM CHLORIDE CONCENTRATION IN THE GRADIENT NEEDED TO ELUTE AROMATIC CHOLINE ESTERASE ACTIVITY FROM FPLC ANION-EXCHANGE COLUMN

Plant species	*NaCl (M)*
Barbarea intermedia	0.14–0.17
Brassica nigra	0.14–0.18
Sinapis alba	0.19–0.22
Brassica napus cv. Gulliver	0.23–0.26
Brassica campestris	0.24–0.27
Brassica napus cv. Line	0.24–0.28

injection loop (maximum 1000 μl), successive analyses were performed, and the purified proteins were collected in the same tubes.

The division of the esterases into apparently three groups (Table II) has no explanation yet. The enzymes from *B. napus* and *B. campestris* eluted at the same sodium chloride concentration, whereas the enzyme from *B. nigra* eluted at a much lower concentration. The esterases also behaved differently using chromatofocusing (Fig. 2). The *B. intermedia* esterase was further separated into two isoenzymes. These qualitative differences among the enzymes from the different plants follow differences in the contents of different aromatic choline esters, although the significance thereof is unknown.

Fig. 2 shows selected results obtained by FPLC chromatofocusing of aromatic choline esterases (isoenzymes) from *B. intermedia* (a; fractions 25 and 31) and chromatography after addition of an equal amount of enzyme from *B. campestris*. The enzyme from *B. campestris* (b; fraction 38) is well separated from the two aromatic choline esterases from *B. intermedia* (b; fractions 25 and 31). The technique applied resulted in highly purified enzymes, as revealed by comparison of the curves for protein and enzyme activity (Fig. 2).

Table III shows a comparison of results obtained by purification and characterization of aromatic choline esterases from seedlings of six cruciferous plants.

FPLC ion-exchange chromatography yielded a 3–16-fold purification and nearly quantitative recoveries (67–100%) of the injected activity. This result is in agreement with our previous findings in experiments with the *S. alba* enzyme[11]. The only reported purification of the enzyme is that of Tzagoloff[12], who achieved a 17-fold purification using acetone and ammonium sulphate precipitation of sinapine esterase from *B. hirta* Moench. In newer investigations of the enzyme[13,17] no values for the purification were given.

Ion-exchange chromatography was performed at pH 7.60, which is about three pH units higher than the p*I* of the aromatic choline esterase (Table III). The pH gradient created during FPLC chromatofocusing and the elution of the aromatic choline esterases were reproducible. This allows the determination of relative p*I* values of the enzymes, which can be used in a qualitative comparison of the enzymes from different sources (Fig. 2 and Table III). A comparison of the relative p*I* values obtained by FPLC chromatofocusing and the relative p*I* values obtained by isoelectric focusing has also been performed for other plant enzymes[18].

The results in Table III confirm that the aromatic choline esterases are not

TABLE III

PURIFICATION AND CHARACTERIZATION OF AROMATIC CHOLINE ESTERASES FROM CRUCIFERS

Characterization as revealed from Fig. 2 showing chromatofocusing; this last purification step yielded practically pure enzymes.

Plant species	*Activity (U)*					*Protein (mg)*			
	Crude extract	*$(NH_4)_2SO_4$*	*G-75 filtration*	*FPLC ion exchange (best prep.)*	*FPLC ion exchange (rest)*	*Crude extract*	*$(NH_4)_2SO_4$*	*G-75 filtration*	*FPLC ion exchange (best prep.)**
B. napus cv. Line	2.83	2.25	0.814	0.300	0.036	85.2	36.1	0.956	0.099
B. campestris	1.53	0.996	0.277	0.077	0.027	72.3	34.4	1.16	0.078
B. napus cv. Gulliver	4.84	3.72	1.27	0.199	0.030	48.9	19.0	0.812	0.048
Sinapis alba§§	1.43	1.16	0.326	0.072	0.027	33.3	10.3	0.38	0.024
B. nigra	2.47	1.33	0.406	0.128	0.063	84.2	30.0	0.878	0.216
Barbarea intermedia	0.493	–	0.282	0.162	0.008	25.6	–	1.21	0.180

* FPLC: A_{280} measured.
** Defined as increase in specific activity per step.
*** Percentage activity found of that applied to column.
§ Only relative values estimated from FPLC chromatofocusing.
§§ Molecular weight determined to be 40000 daltons (gel filtration).

TABLE IV

KINETIC PARAMETERS FOR AROMATIC CHOLINE ESTERASES FROM CRUCIFERS

The data were obtained under conditions where no hysteresis effects were observed. None of the examined enzymes showed any appreciable activity towards the substrate 3,4-dimethoxybenzoylcholine (hesperalin). The results for K_m are in μM; the V_{max} values are relative to V_{max} for sinapine.

Plant species	*Substrate*							
	Sinapine		*Isoferuloyl-choline*		*p-Coumaroyl-choline*		*p-Hydroxy-benzoylcholine*	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Barbarea intermedia	23.9	100	23.3	93	3.1	53	n.d.	n.d.
Brassica nigra	6.0	100	1.1	38	4.3	41	5.5	20
Sinapis alba	4.0	100	3.2	30	0.66	42	134	23
Brassica napus cv. Gulliver	11.5	100	3.9	40	3.9	36	52.5	12
Brassica campestris	14.6	100	5.8	39	8.2	30	n.d.	n.d.
Brassica napus cv. Line	10.4	100	n.d.	n.d.	3.7	37	n.d.	n.d.

Specific activity (U/mg protein)				*Purification** (fold per step)*			*Recovery (%)*** [FPLC ion exchange (best prep.)]*	*pI§*
Crude extract	*$(NH_4)_2SO_4$*	*G-75 filtration*	*FPLC ion exchange (best prep.)*	*$(NH_4)_2SO_4$*	*G-75 filtration*	*FPLC ion exchange (best prep.)*		
0.033	0.062	0.851	3.03	1.9	13.7	15.5	79	4.3
0.021	0.029	0.239	0.992	1.4	8.2	10.7	70	4.3
0.099	0.196	1.56	4.15	2.0	8.0	6.6	67	4.3
0.043	0.113	0.858	3.000	2.6	7.6	5.9	90	4.3
0.029	0.044	0.462	0.593	1.5	10.4	4.7	76	4.4
0.091	–	0.233	0.900	–	–	3.1	100	5.0 and 4.7

identical, and that different isoenzymes exist in *B. intermedia*. The combination of ion-exchange chromatography and chromatofocusing is valuable for obtaining reliable information about the enzyme activity in various plant species for comparison studies. The stability of the system needs to be high in order to observe minor differences. On the other hand, the same combination of methods can also be used to obtain a larger amount of virtually pure enzyme for more detailed studies, including kinetic studies, for characterization purposes.

Results from enzyme kinetics, based on the use of different substrates, are shown in Table IV. Some combinations of enzyme and substrate concentrations gave rise to hysteresis effects. These combinations were avoided in experiments used to obtain K_m and V_{max} values. The kinetic parameters also confirm that the enzymes for the six sources are different.

It is remarkable that for the enzyme in *S. alba*, *p*-hydroxybenzoylcholine, which is present in the seeds in large amounts, is the poorest substrate. The reason could be that the enzyme isolated is not responsible for the catabolism of *p*-hydroxybenzoylcholine, which occurs much later than the degradation of sinapine in *Sinapis* seedlings[7,10].

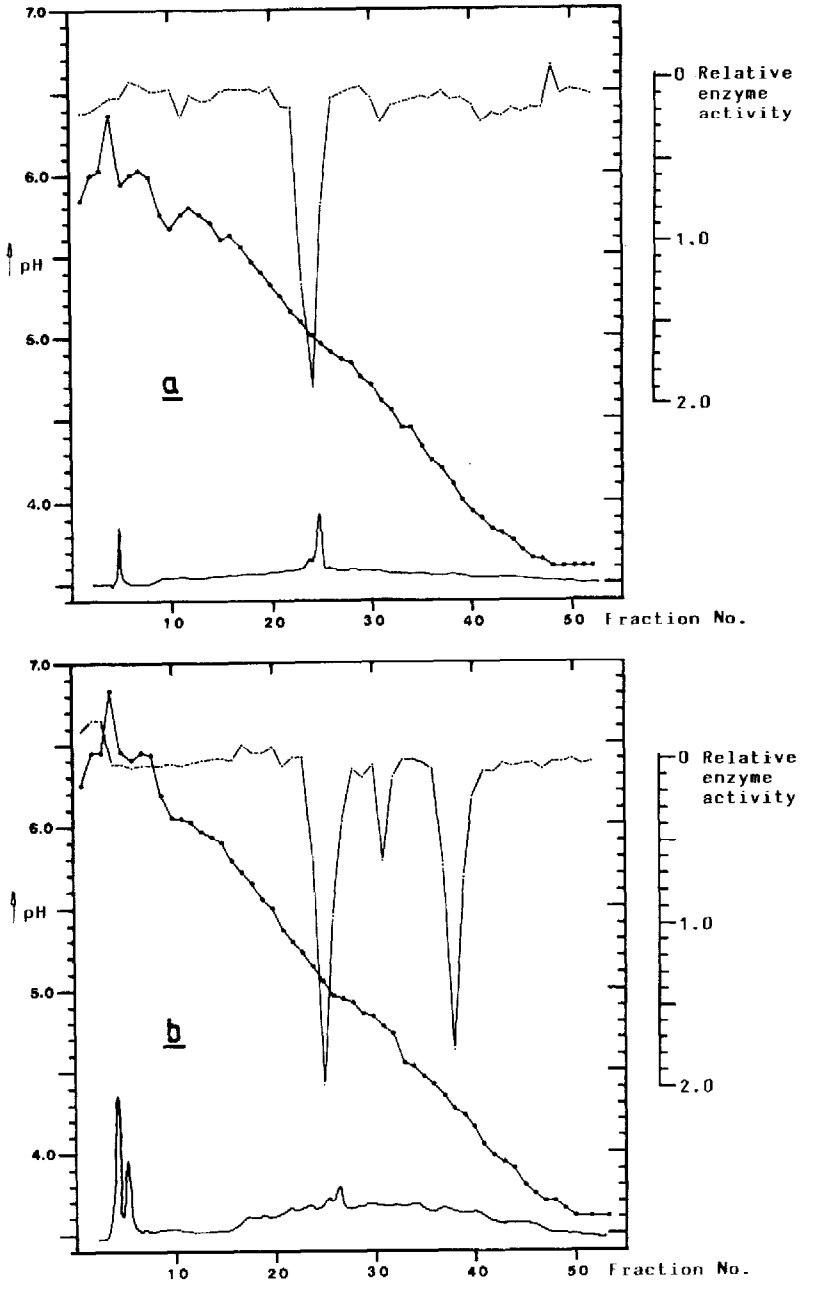

Fig. 2. (a) Chromatofocusing of aromatic choline esterase isoenzymes from *Barbarea intermedia* and (b) chromatography of a mixture with the enzyme from *Brassica campestris*. Solid line (●), pH of the eluates; broken line (●), results from assay; a valley shows activity of aromatic choline esterase. ——, A_{280}.

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