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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

RAPID CHROMATOFOCUSING OF PLANT ENZYMES

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SUMMARY

High-performance liquid chromatography was used to purify four different enzymes extracted from tobacco leaves. Phenylalanine ammonia-lyase (E.C. 4.3.1.5) and three O-methyltransferases (S-adenosyl-L-methionine: catechol O-methyltransferases, E.C. 2.1.1.6) were subjected to high-performance chromatofocusing. Parameters affecting the resolution of chromatography and the recovery of enzyme activity were investigated. The speed and high resolving power of chromatofocusing are major advantages for analytical or preparative purposes. The absorbance at 280 nm of chromatographic fractions was shown to arise mainly from small molecules and was not a measurement of protein concentration as indicated by subsequent high-performance size exclusion chromatography. Electrophoretic analysis of the active fractions on sodium dodecyl sulphate–polyacrylamide slab gels demonstrated the high degree of purification achieved by chromatofocusing.

INTRODUCTION

Many plant enzymes have high specific catalytic activities and are present in only minor amounts in plant extracts. Therefore, it is often difficult to achieve their purification to homogeneity by conventional means. Recently, techniques of high-performance liquid chromatography (HPLC) have become available for the separation of proteins[1,2]. In a previous paper we described the use of high-performance ion-exchange chromatography to purify plant enzymes[3]. After this step of HPLC a few contaminants remained in enzyme preparations and we report here our attempts to improve the purification of enzymes by high-performance chromatofocusing. Four enzymes were subjected to HPLC chromatofocusing. They are all involved in the phenylpropanoid pathway, which leads to various compounds derived from phenylalanine, namely coumarins, flavonoids and lignin. The first enzyme is phenylalanine ammonia-lyase (E.C. 4.3.1.5; PAL), which catalyses the deamination of phenylalanine to cinnamic acid. The three others are O-methyltransferases (S-adenosyl-L-meth-

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ionine: catechol O-methyltransferases, E.C. 2.1.1.6; OMT), which methylate *o*-diphenolic substrates, particularly caffeic and 5-hydroxyferulic acids, which are precursors of lignin. The three OMTs from tobacco leaves are separable by ion-exchange chromatography[3] and have different substrate specificities[4].

Chromatofocusing is a new ion-exchange technique, which was first described by Sluyterman and co-workers[5–7]. Negatively charged proteins are adsorbed on the ion exchanger and then eluted by a pH gradient generated in the column as the eluting buffer titrates the ion exchanger. Proteins elute at a pH near their p*I*[8]. Recently, chromatofocusing has been adapted to HPLC[9,10] and some parameters have been studied using commercially available purified proteins as markers[9]. Here the parameters affecting the resolution and recovery of enzyme activity were investigated. Experimental conditions were optimized for each enzyme and allowed the separation of several enzymatic forms. Attempts to use chromatofocusing for enzyme purification at a preparative scale are also reported.

EXPERIMENTAL

Plant material

Tobacco plants were grown under controlled conditions as described previously[4]. PAL was extracted from leaves inoculated with tobacco mosaic virus 48 h before harvest. OMTs were obtained after an infiltration procedure already described[3].

Enzyme extraction

The extraction procedure for OMTs was described earlier[4]. For PAL the procedure was essentially the same except that 0.25 *M* potassium phosphate buffer (pH 7.8) was used.

Purification steps preceding chromatofocusing

Proteins were precipitated with ammonium sulphate between 30 and 55% saturation for the preparation of PAL or 40 and 75% saturation for OMTs. The protein fraction was resuspended in 70 ml of sodium phosphate buffer (pH 7.5) and chromatographed successively on a 35 × 4 cm I.D. Sephadex G-25 column (Pharmacia), a 90 × 7 cm I.D. ultrogel AcA34 column (IBF) and a 15 × 4.5 cm I.D. DEAE-cellulose column (Schleicher and Schüll). The DEAE-cellulose column was eluted first with 300 ml of 40 m*M* phosphate buffer (pH 7.7), followed by 2 l of a linear gradient from 50 to 110 m*M* of the same buffer for the separation of OMTs and from 50 to 150 m*M* for the preparation of PAL. Each enzyme was further purified by high-performance ion-exchange chromatography as described previously[3].

Assay of enzyme activity

PAL activity. Volumes of 50 μl of enzymatic fractions were incubated in the presence of 1 ml of 20 m*M* phenylalanine in 0.1 *M* borate buffer (pH 8.8) for 22 h at 37°C. The rate of conversion of phenylalanine into cinnamic acid was determined by measuring the absorbance at 290 nm.

OMT activity. The standard assay mixture consisted of 1 ml of 50 μ*M* tritiated S-adenosyl-L-methionine (3.7 kBq per assay) and 3 m*M* caffeic acid or 1.3 m*M* cat-

echol as *o*-diphenolic substrate. The volumes of enzyme solution and incubation time at 37°C depended on the level of enzyme activity and are indicated in the text. The enzymatic reaction was stopped with two drops of 9 *N* sulphuric acid. The reaction product was extracted and its radioactivity measured as described previously[4].

High-performance liquid chromatography equipment

Buffers and reagents were of analytical-reagent grade. All buffers were filtered through 0.22-μm filters and degassed under vacuum. Protein solutions were filtered through membranes of cellulose acetate (Celotate, Millipore). The Pharmacia fast protein liquid chromatography (FPLC) system was used for high-performance ion-exchange chromatography on a pre-packed Mono Q HR 5/5 (50 × 5 mm I.D.) column (Pharmacia) and chromatofocusing on a Mono P HR 5/20 column (Pharmacia). The FPLC system consisted of two P-500 pumps, a GP-250 gradient programmer, a V-7 injection valve, two V-8 valves, a 50-ml Superloop, a UV-1 monitor with HR flow cell, an REC-482 chart recorder and a FRAC 100 fraction collector. High-performance size-exclusion chromatography was carried out on an LKB TSK G3000 SW column using an LKB HPLC system consisting of a 2150 HPLC pump, a 2238 Uvicord SII and a 2112 Redirac fraction collector.

Chromatofocusing

The equilibrating buffers were 25 m*M* histidine · HCl (pH 6.2) and 25 m*M* methylpiperazine · HCl (pH 5.7 or 5.3). Samples were dialysed against the equilibrating buffer before loading on the Mono P column. Elution was carried out with Polybuffer 74 (Pharmacia), which was diluted 10-fold, adjusted to pH 3.7 or 4.0 with 1 *N* hydrochloric acid and injected on to the column through the 50-ml Superloop. Every tenth fraction was used to measure the pH value. Fractions of 340 μl were collected in 20 μl of 1 *M* Tris (pH 10.9) containing 850 μ*M* phenylalanine for chromatography of PAL. Fractions of 170 μl were collected for preparations of OMTs in 10 μl of 1 *M* Tris (pH 8.3) for OMT I and of 1 *M* Tris (pH 8.0) for OMT II and III. After each run the column was washed with successive injections (500 μl each) of 1 ml of 2 *M* sodium chloride and 3 ml of 70% (v/v) acetic acid. After the washing steps the column was equilibrated with at least 30 ml of starting buffer.

Polyacrylamide gel electrophoresis

Protein fractions were analysed by polyacrylamide slab gel electrophoresis according to the method of Laemmli[11]. The experimental conditions have been described previously[3].

RESULTS AND DISCUSSION

Important parameters for chromatofocusing

One of the three O-methyltransferases of tobacco (OMT III) was taken as a marker and subjected to chromatofocusing under various experimental conditions (Fig. 1). The enzyme had been previously purified by a procedure including conventional low-pressure chromatographic steps and one high-performance ion-exchange chromatographic separation, which have been described earlier[3]. The effects of gra-

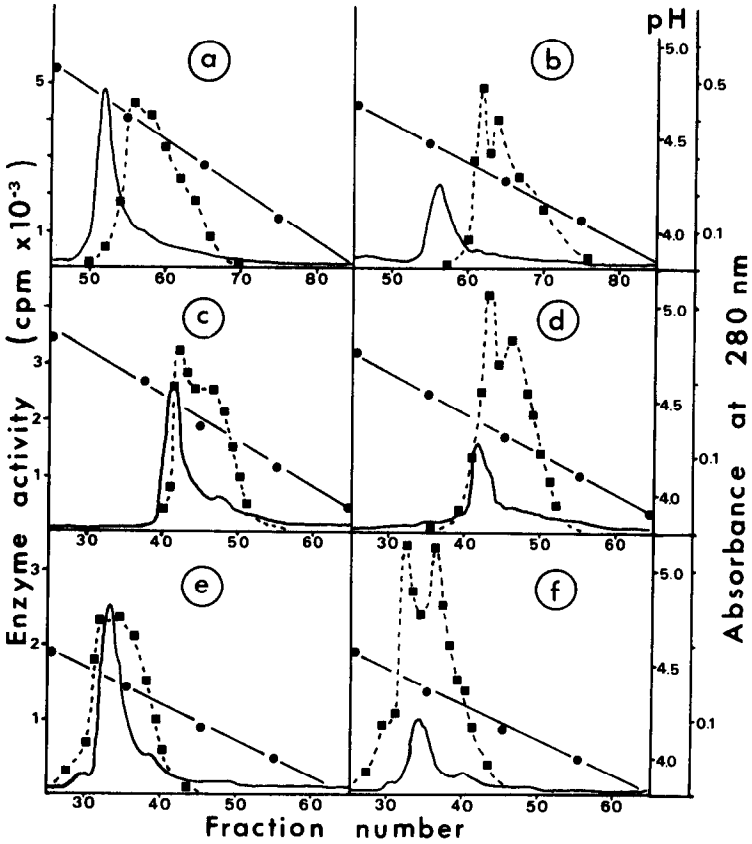

Fig. 1. Chromatofocusing of O-methyltransferase III under various experimental conditions. The column was equilibrated with either 25 m*M* histidine · HCl buffer (pH 6.2) (a and b), 25 m*M* methylpiperazine · HCl (pH 5.7) (c and d) or 25 m*M* methylpiperazine · HCl (pH 5.3) (e and f). A volume of 2 ml of enzymatic solution containing about 0.4 mg of protein was applied to the column in each experiment. Elution was carried out with Polybuffer 74 (pH 3.7) at a flow-rate of 1 ml/min in the experiments illustrated in a, c and e and 0.3 ml/min in b, d and f. The collected fractions consisted of a 3-ml fraction followed by 0.34-ml fractions. A volume of 20 µl was withdrawn to assay OMT activity and was incubated for 30 min in the presence of 1.3 m*M* catechol as diphenolic substrate (■---■). Absorbance (———) was monitored at 280 nm and the pH gradient is represented by ●—●.

dient slope and flow-rate on resolution were investigated. The mono P column was equilibrated with either 25 m*M* histidine · HCl buffer (pH 6.2) (Fig. 1a and b) or 25 m*M* methylpiperazine · HCl buffer adjusted to pH 5.7 (Fig. 1c and d) or pH 5.3 (Fig. 1e and f) and eluted with Polybuffer (pH 3.7) in all instances. As expected, different pH gradients were generated but the activity profiles were almost unaffected. Flow-rate appears to be a more important factor: for every gradient one broad peak of activity was detected at a flow-rate of 1 ml/min, whereas two peaks were resolved at 0.3 ml/min. Hence decreasing the flow-rate of the eluent increased the resolution. The absorbance at 280 nm was monitored and one major peak was revealed. Surprisingly, its position depended on the pH gradient and it eluted before the enzyme activity peak in profiles a and b or simultaneously in profiles e and f. A subsequent analysis

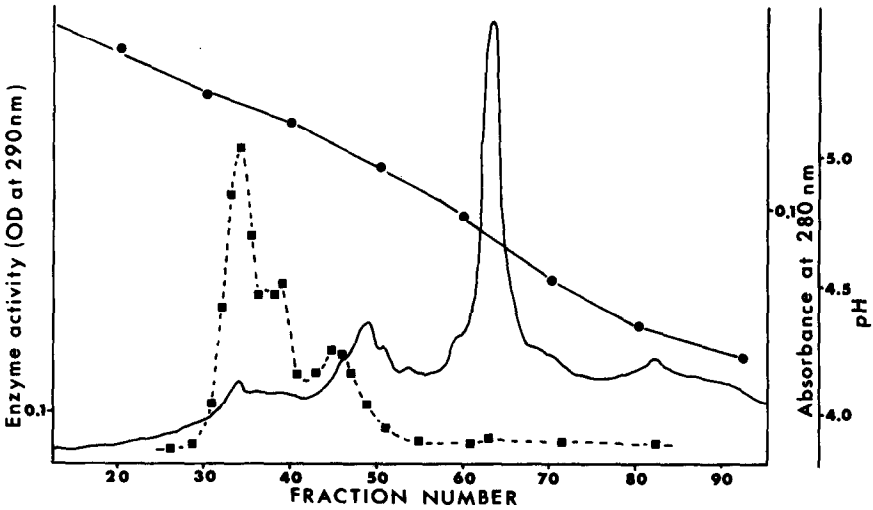

Fig. 2. Chromatofocusing of a fraction containing phenylalanine ammonia-lyase. The Mono P column was equilibrated with 25 m*M* histidine · HCl (pH 6.2). A 7-ml volume of protein solution containing about 2 mg of protein was injected on to the column at a flow-rate of 1 ml/min. Elution was performed at a flow-rate of 0.5 ml/min with Polybuffer 74 (pH 4.0). After the first 3 ml, fractions of 0.34-ml were collected in 20 μl 1 *M* Tris (pH 10.9) containing 850 μM phenylalanine; 50 μl were withdrawn and used to assay PAL activity (■---■) as described under Experimental. Absorbance (———) was monitored at 280 nm and the pH of every tenth fraction was measured (●—●).

by high-performance size exclusion chromatography demonstrated that the absorbance in this peak arose predominantly from small molecules (data not shown).

Chromatofocusing of phenylalanine ammonia-lyase and O-methyltransferases

As already pointed out, enzymatic proteins are eluted during chromatofocusing at a pH value near their p*I*. This may have dramatic effects on the recovery of enzyme activity and, in fact, no activity at all was recovered in early experiments of chromatofocusing with PAL and OMT I. For OMT II and III 50% of the activity was lost. By adding a small volume of Tris buffer to the collection tubes in order to increase rapidly the pH of the collected fractions, the recovery of enzyme activity reached about 40% for OMT I and 80–95% for OMT II and III. The addition of phenylalanine, the substrate of PAL, in Tris buffer increased the yield of this enzyme activity to 20–40%. Figs. 2–5 show the activity profiles, the absorbance patterns and the pH gradients obtained during chromatofocusing of PAL and OMT I, II and III, respectively. The experimental conditions were chosen taking into account two opposite requirements: a flow-rate as low as possible to obtain a high resolution and an elution time as short as possible to reduce the loss of enzyme activity. The four enzymes eluted at mean pH values of 5.2, 4.7, 4.3 and 4.25 for PAL and OMT I, II and III, respectively. These pH values varied by 0.1–0.2 pH unit, depending on the total amount of protein and also on the proportion of enzyme molecules in the protein mixture loaded on the column. From more than 50 independent experiments it was established that 1–4 mg of protein containing about 0.1 mg of enzymatic protein represents the maximum amount that can be chromatographed with high

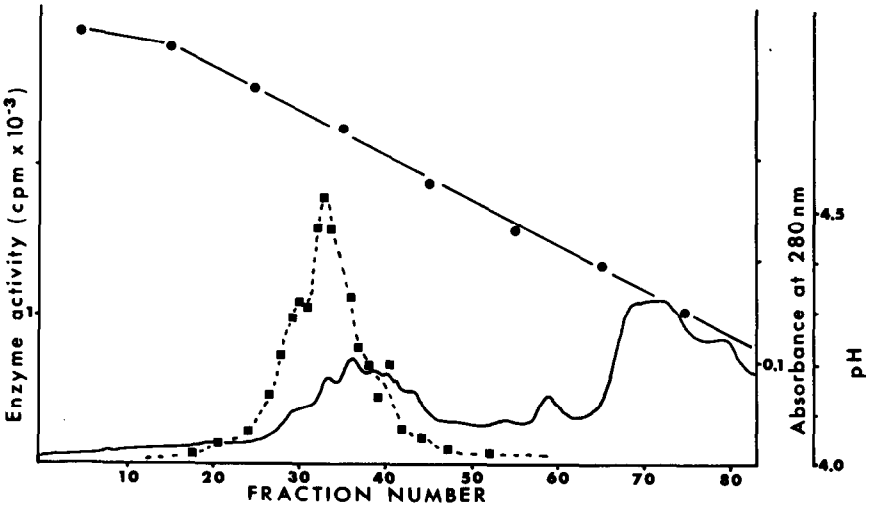

Fig. 3. Chromatofocusing of O-methyltransferase I. The equilibrating buffer was 25 m*M* histidine · HCl (pH 6.2). A 6.5-ml volume of enzymatic solution containing 3 mg of protein was injected on to the column. Elution with Polybuffer 74 (pH 4.0) was performed at a flow-rate of 0.5 ml/min. After an elution volume of 6 ml, 170-μl fractions were collected in 10 μl of 1 *M* Tris (pH 8.3); 10 μl were incubated for 15 min with 3 m*M* caffeic acid as diphenolic substrate (■---■). The absorbance at 280 nm (———) and pH gradient (●—●) are shown.

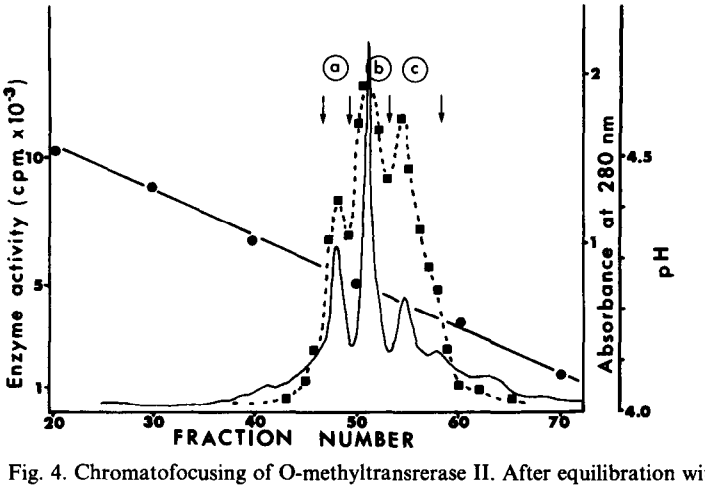

Fig. 4. Chromatofocusing of O-methyltransrerase II. After equilibration with 25 m*M* methylpiperazine · HCl (pH 5.7), 12 ml of enzyme solution containing about 4 mg of protein were loaded on to the column. Polybuffer 74 (pH 3.7) was used for elution at a flow-rate of 0.5 ml/min; 9 min after the beginning of elution, 200-μl fractions were collected in the presence of 10 μl of 1 *M* Tris (pH 8.0) and 5 μl were assayed for 15 min with 1.3 m*M* catechol as diphenolic substrate (■---■). The absorbance (———) at 280 nm and pH gradient (●—●) are presented. Fractions between vertical arrows were pooled and analysed further as peaks a, b and c.

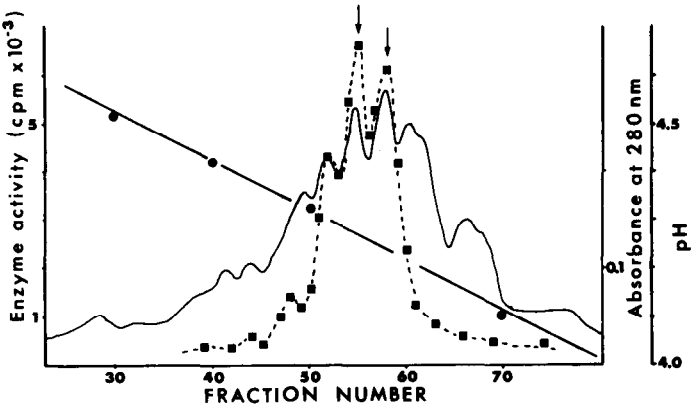

Fig. 5. Chromatofocusing of O-methyltransferase III. A 3-ml volume of enzymatic solution containing 1.5 mg of protein was injected on to the column, which had been equilibrated with 25 m*M* methylpiperazine · HCl buffer (pH 5.7). Other experimental conditions as in Fig. 4. The pH gradient (●—●), absorbance at 280 nm (———) and enzyme activity (■---■) are shown.

resolution. It is noteworthy that the volume of sample is not a limiting factor, as up to 12 ml (Fig. 4) were loaded on the column without any loss of resolution.

Several peaks of activity were detected for every enzyme. This suggests the occurrence of different forms of enzymes with very close elution pH values; for example, peaks marked by an arrow in Fig. 5 are separated by only 0.05 pH unit. The number and the relative amounts of these different enzymatic forms vary for a given enzyme from one preparation to another. In an attempt to purify these different forms of OMT II, the active fractions containing peaks a, b and c of Fig. 4 were pooled and subjected to a second chromatofocusing. The elution patterns of fractions a, b and c are presented in Fig. 6 and indicate that whereas b is still heterogenous, one form of enzyme is predominant in a and c. This suggests that the separation of each form should be possible by successive runs on the Mono P column, provided that the enzyme is sufficiently stable and available in a sufficient amount. Further,

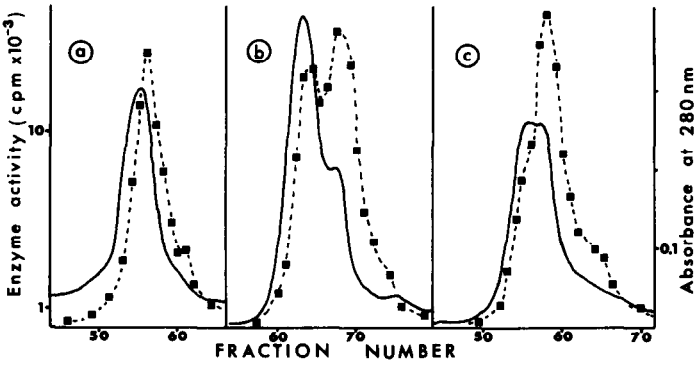

Fig. 6. Chromatofocusing of forms a, b and c of O-methyltransferase II partially resolved in the experiment shown in Fig. 4. After dialysis against equilibrating buffer, pooled fractions (see Fig. 4) were injected on to the column. Experimental conditions as in Fig. 4 except that enzyme activity was elaluated by incubating 10 μl of each fraction for 50 min. Enzyme activity (■---■) and absorbance at 280 nm (———) are shown.

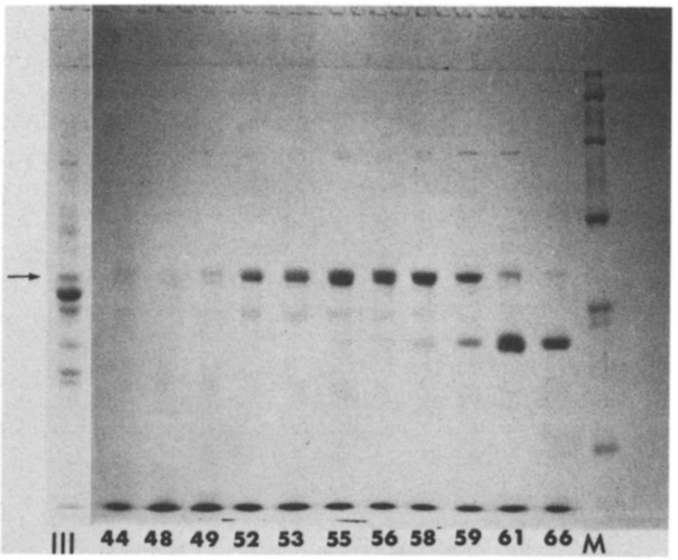

Fig. 7. Polyacrylamide gel electrophoresis of fractions obtained after chromatofocusing of O-methyltransferase III. Electrophoresis and staining of gels were carried out as described earlier[3]. Lane III shows the protein pattern of the protein solution that was loaded on the column. Lanes 44–66 show the protein patterns of the corresponding fractions obtained from the chromatofocusing experiment illustrated in Fig. 5. Molecular-weight-marker proteins (M) were α_2-macroglobulin (170 kd), phosphorylase b (97.4 kd), glutamate dehydrogenase (55.4 kd), lactate dehydrogenase (36.5 kd) and trypsin inhibitor (20.1 kd).

these results indicate that equilibration between the different enzymatic forms does not take place at a significant rate.

Analysis of elution patterns by gel electrophoresis and high-performance size-exclusion chromatography

Fractions obtained after chromatofocusing of the four different enzymes were analysed by electrophoresis on polyacrylamide gels in the presence of SDS. As an example, Fig. 7 shows the photograph of a slab gel after electrophoresis of fractions obtained by chromatofocusing of OMT III. Lane III shows the protein pattern of the partially purified enzyme solution that was loaded on the column. Lanes 44–66 show the protein patterns of the corresponding fractions obtained from the chromatofocusing experiment illustrated in Fig. 5. Comparison of lane III and lanes 52–59, which correspond to the most active fractions, clearly demonstrates the improvement in purification brought about by chromatofocusing. For instance, the major contaminant in the initial enzyme preparation loaded on to the column (lane III) was completely eliminated by chromatofocusing. Another advantage of the technique is the high concentration of protein fractions after elution, as evidenced for the enzymatic protein (shown by the arrow) in active fractions and for a proteinaceous contaminant eluted in fractions 61 and 66. In the active fractions corresponding to the three distinct peaks of activity (Fig. 5) the band of enzyme subunit migrated at the same position, indicating that the different forms of the enzymes have the same molecular weight. This was found to be the case for each enzyme.

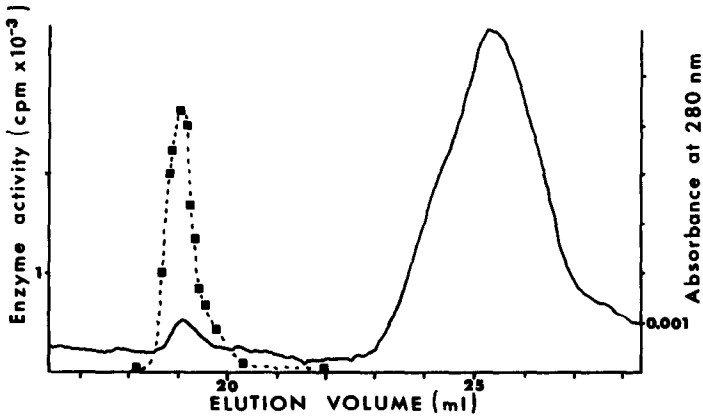

Fig. 8. High-performance size-exclusion chromatography of the active fractions obtained after chromatofocusing of O-methyltransferase III. A volume of 200 μl was loaded on a TSK 3000 SW column, which was eluted with 0.1 *M* sodium phosphate buffer (pH 6.9) containing 0.2 *M* NaCl. Enzyme activity (■---■) was assayed by incubating 20 μl of each 110-μl fraction for 2 h with 1.3 m*M* catechol as diphenolic substrate. The absorbance (———) was monitored at 280 nm.

We tentatively determined the amount of enzymatic protein by densitometry of the stained gels and comparison of staining intensities of the bands with those of standard proteins. The results were not in agreement with the amounts of protein expected on the basis of the absorbance of the chromatographic fractions. In order to discover the origin of these discrepancies, active fractions from chromatofocusing were pooled and analysed by high-performance size-exclusion chromatography. Activity and absorbance curves of a typical experiment are presented in Fig. 8. A major peak of absorbance was completely separated from the peak of enzyme activity, the absorbance of which was 10–30 times lower than that of the major peak as calculated from the ratios between peak areas. The elution volume and the broadness of the major peak of absorbance suggest that it contains a mixture of molecules of low molecular weight (in the range of a few thousand daltons). This material migrated with the front during electrophoresis on polyacrylamide gels and was present in all fractions from chromatofocusing (Fig. 7). These small molecules should be mainly Polybuffer molecules that coelute with protein and interfere with absorbance measurement. This was unexpected, because no absorbing material was eluted from the Mono P column when a blank run was performed (data not shown).

In this work, chromatofocusing has been successfully applied to the HPLC system for four distinct enzymes. Its resolving power was strikingly demonstrated by the detection and partial separation of different forms of enzymes that have been shown to have the same molecular weight on SDS–polyacrylamide gels. This technique also offers the advantages of speed and preparative capabilities. The major shortcoming was found to be the presence, in active fractions, of Polybuffer molecules that interfere with the detection of proteins by their absorbance at 280 nm. The separation of proteins from Polybuffer can be easily achieved by high-performance size-exclusion chromatography and results in highly purified enzymes.

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