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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

PURIFICATION OF PLANT ENZYMES BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography (HPLC) was used to purify catalytically-active enzymes present in minor quantities in plant material. The three O-methyltransferases (S-adenosyl-L-methionine:catechol O-methyltransferases, E.C. 2.1.1.6) of tobacco leaves were subjected to high-performance ion-exchange chromatography. Excellent recovery of enzyme activity (70–100%) was obtained. HPLC was tentatively used at both analytical and preparative scales. As an analytical tool HPLC offered major advantages over conventional low-pressure ion-exchange chromatography in both speed and resolving power. For preparative purposes however, pre-purification of plant extracts by conventional means was necessary before HPLC. Purification achieved by HPLC was evidenced by electrophoretical analysis of the active fractions on sodium dodecyl sulphate–polyacrylamide slab gels.

INTRODUCTION

The recent availability of rigid chromatographic supports which allow high flow-rates, has enabled the separation of proteins by high-performance liquid chromatography (HPLC) based on the same criteria as those used in conventional liquid chromatography. These criteria are differences in isoelectric point, differences in net charge at different pH values, or differences in apparent molecular weight. HPLC of proteins is a rapidly expanding field but up to now there are only a few examples where it has been used to purify active enzymes[1–7]. In most cases the enzymatic activities extracted from materials other than plants were very stable and easy to test on-line. Plant enzymes are particularly difficult to purify because plant cell extracts contain numerous low-molecular-weight compounds (phenols, tannins) which are easily oxidised and polymerised during extraction. These compounds adsorb on the chromatographic supports in a non-reversible manner and reduce the lifetime of the columns. Thus, it is not possible to analyse large amounts of crude plant cell extracts

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on HPLC columns. We have overcome these difficulties by pre-purification of plant extracts by conventional means: soluble proteins extracted from tobacco leaves were fractionated by ammonium sulphate precipitation, low-pressure chromatographic steps including size exclusion on Ultrogel Ac34 and ion-exchange on DEAE-cellulose. The resulting enzymatic fractions have been subjected to high-performance ion-exchange chromatography (HPIEC) on a Mono Q column. Parameters affecting HPIEC have been studied recently using commercially available purified proteins as markers[8,9]. The elution pattern of the enzymatic proteins was followed by measurement of enzyme activity. The enzymes investigated ((S-adenosyl-L-methionine:catechol O-methyltransferases, E.C. 2.1.1.6) all catalyse the O-methylation of various *o*-diphenolic compounds among which caffeic and 5-hydroxyferulic acids, which are intermediates in the biosynthesis of lignin. Three distinct enzymes with different substrate specificities had been characterized previously from tobacco leaves[10].

In the present paper two aspects were investigated. First, we compared the performances of conventional DEAE-cellulose and HPIEC using tobacco O-methyltransferases as markers. Secondly, we examined the usefulness of HPIEC as a further purification step for O-methyltransferases after their separation by DEAE-cellulose chromatography.

MATERIALS AND METHODS

Plant material

Tobacco plants were grown under controlled conditions as described previously[10]. Fully expanded leaves were detached from the plants, their midribs were removed and the resulting half-leaves were infiltrated with water under vacuum. They were then floated on water for three days, harvested, frozen in liquid nitrogen and stored at −80°C.

Enzyme extraction

The extraction procedure was described earlier[10].

Purification steps preceding HPLC

The protein fraction that precipitated between 40 and 75% saturation with ammonium sulphate was dissolved in 70 ml sodium phosphate buffer pH 7.5, and chromatographed successively on a 35 × 4 cm Sephadex G-25 column (Pharmacia), a 90 × 7 cm Ultrogel Ac34 column (IBF) and a 15 × 4.5 cm DEAE-cellulose column (Schleicher and Schüll).

High-performance chromatography equipment

Chromatography materials (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 (Pharmacia, Uppsala, Sweden) was used throughout the study and consisted of two P-500 pumps, a GP-250 gradient programmer, a V-7 injection valve, a 50-ml superloop, a UV-1 monitor with HR flow cell, and a REC-482 chart recorder. For HPLC a prepacked Mono Q HR 5/5 (50 × 5 mm I.D.) column (Pharmacia) was used.

The salt gradient was made by mixing two buffers A and B delivered by the two pumps controlled by a programmer. Buffer A was 25 m*M* triethanolamine–HCl pH 7.7 and buffer B was 25 m*M* triethanolamine–HCl containing 1 *M* sodium chloride. At the end of each run the column was washed automatically with buffer B and then equilibrated with buffer A.

Assay of enzyme activity

The standard assay procedure for O-methyltransferase activity was carried out with 1 ml of 50 μM tritiated S-adenosyl-L-methionine (3.7 kBq/assay), caffeic acid or catechol as *o*-diphenolic substrate (at a concentration as indicated in the text) and varying volumes of enzyme solution. After incubation at 37°C (1 h for DEAE-cellulose fractions, 30 min for HPLC fractions) the reaction was stopped with two drops of 9 *N* sulphuric acid. The reaction products were extracted and their radioactivity measured as described previously[10].

Estimation of the amount of protein

The quantity of proteins was evaluated, based on the method of Bradford[11]. A volume of 1 ml of a solution (50 mg/l) of Serva Blue G (Serva, Heidelberg) containing 4.7% (v/v) ethanol and 8.5% (w/v) phosphoric acid was added to the protein solution (5–50 μl). The absorption at 595 nm was used to calculate the amount of protein using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Protein fractions were analysed by polyacrylamide slab gel electrophoresis according to the method of Laemmli[12] using a 5% stacking gel and a 10% separating gel. Before electrophoresis 0.1 volume of a solution containing 20% sodium dodecyl sulphate (SDS), 20% β-mercaptoethanol and 40% glycerol was added to the samples which were boiled for 1 min.A volume of 20 μl was subjected to electrophoresis first at 25 mA until a voltage of 120 V was reached and then at constant voltage. Gels were stained with a solution of 0.15% (w/v) Serva G Blue in methanol–water–acetic acid (45:45:9, v/v/v) for 0.5 h and destained in methanol–water–acetic acid (5:87.5:7.5, v/v/v).

RESULTS AND DISCUSSION

Comparison of the separation of the three enzymes obtained by conventional or high-performance liquid chromatography

The three enzymes were resolved by elution on the DEAE-cellulose column (Fig. 1). The elution was performed with 300 ml of 40 m*M* phosphate buffer pH 7.7, followed by 2 l of a linear gradient from 50 m*M* to 110 m*M* of the same buffer. As expected from previous work[10], caffeic acid (3 m*M*) is methylated by the three enzymes whereas catechol (100 μM) is poorly methylated by O-methyltransferase I. The different affinities of the three enzymes for catechol and caffeic acid are useful to distinguish enzymes I and II when they are poorly separated as illustrated in Fig. 2. Fig. 2 presents the elution profiles of the three enzymes obtained by HPIEC of a plant extract after the Ac34 Ultrogel chromatographic step. The slope of the elution gradient was steep (20 m*M* sodium chloride per min) and the analysis lasted only 15

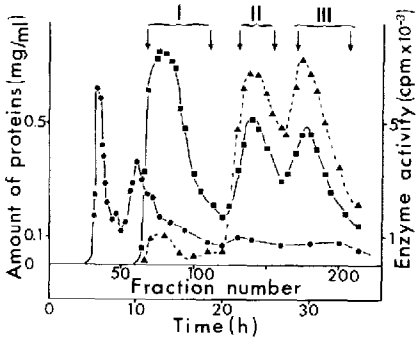


Fig. 1. Elution profiles obtained by chromatography on DEAE-cellulose. Pooled active fractions (440 ml) obtained from chromatography on Ac34 Ultrogel and containing 0.5 g of protein were loaded onto the column at a flow-rate of 1 ml/min. The column was washed with 300 ml of 40 m*M* sodium phosphate buffer pH 7.7, then eluted with a linear gradient of 50 to 110 m*M* sodium phosphate pH 7.7. During washing and elution of the column the flow-rate was 1 ml/min and fractions of 10 ml were collected. The protein content of the fractions (●—●) was estimated as described in Materials and Methods. Enzymatic activity was determined by assaying 100 μl of each fraction with 3 m*M* caffeic acid (■—■) or 100 μM catechol (▲--▲) as substrates. Fractions between vertical arrows were pooled and used further as O-methyltransferases I, II and III.

min. Under these conditions the enzymes were incompletely separated but when the slope of the gradient was less steep (5 m*M* sodium chloride/min) (Fig. 3) the resolution was excellent and the three enzymes were better resolved after 35 min than after a 35-h chromatographic analysis on a DEAE-cellulose column (Fig. 1).

In addition to its speed and resolving power, HPLC offers the great advantage that the protein solutions eluted from the columns are at a concentration that allows monitoring by absorbance at 280 nm (Figs. 2 and 3). In contrast the fractions eluted from the DEAE-cellulose column were very dilute. Their protein content was estimated by the method detailed in the Materials and Methods section. The elution patterns from the HPLC column (Figs. 2 and 3) show that numerous proteins are eluted by the salt gradient before O-methyltransferases I, II and III. These contam-

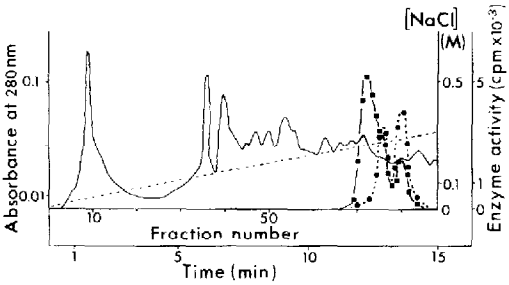


Fig. 2. HPIEC of the active fraction obtained after Ac34 Ultrogel chromatography and containing the three O-methyltransferases. The elution profiles were obtained after rapid elution of a Mono Q HR column. Injection volume: 500 μl containing 0.5 mg of protein. A linear salt gradient of 20 m*M* NaCl/min was used. The initial buffer was 25 m*M* triethanolamine–HCl (pH 7.7) and the final buffer was 25 mM triethanolamine–HCl (pH 7.7) containing 0.3 *M* NaCl. The flow-rate was 1 ml/min and fractions of 0.17 ml were collected. Absorbance was recorded at 280 nm (——). A volume of 50 μl of each fraction was tested for enzymatic activity with 3 m*M* caffeic acid (■—■) or 100 μM catechol (●--●) as substrates.

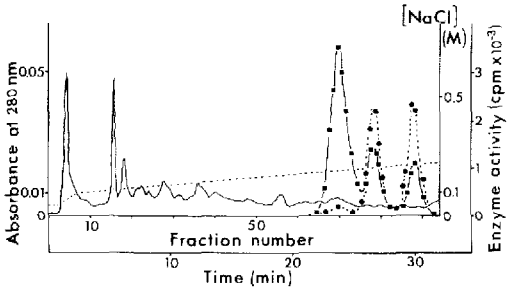


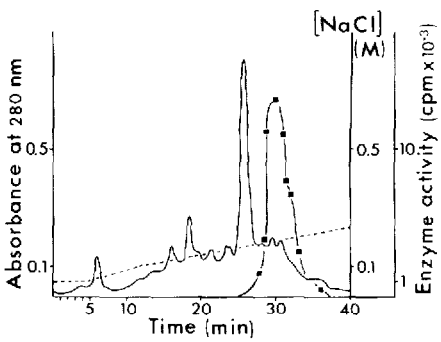
Fig. 3. HPIEC of the active fractions pooled after Ac34 Ultrogel chromatography and containing the three O-methyltransferases. The elution profiles were obtained after elution of a Mono Q HR column under conditions giving high resolution. An amount of 0.5 mg of protein was loaded onto the column. The dotted line represents the change in sodium chloride concentration during elution. Fractions of 0.34 ml were collected. A volume of 0.1 ml of each fraction was tested for O-methyltransferase activity with 3 m*M* caffeic acid (■—■) and 100 μM catechol (●---●). Absorbance (——) was recorded at 280 nm.

inating proteins are not resolved on DEAE-cellulose (Fig. 1) and are probably eluted during loading, washing or at low concentrations of the salt gradient. Thus ion-exchange chromatography appears to be the method of choice to remove many proteins that contaminate the three enzymes after the initial purification steps.

HPLC is a very effective way of performing ion-exchange chromatography. However it should be pointed out that the DEAE-cellulose column used in this study had a loading capacity exceeding 500 mg of protein whereas the maximum capacity of the Mono Q column is 20 mg. Moreover, plant extracts contain many colored compounds which adsorb in a non-reversible manner to the column and reduce its lifetime. Therefore, it would be very costly to chromatograph large amounts of rather crude extracts on HPLC columns. In the experiments illustrated in Figs. 2 and 3 for instance, only 0.5 mg of protein was loaded on the column. Therefore, in the routine preparative procedure we developed HPIEC was performed on the three fractions containing O-methyltransferase activity, obtained by preliminary chromatography on DEAE-cellulose.

Further purification by HPIEC of the O-methyltransferases separated on DEAE-cellulose

In our standard procedure of enzyme purification, active fractions eluted from DEAE-cellulose columns were pooled and their protein content were estimated as described in the Materials and Methods section. The enzyme solutions contained 50–200 μg of protein per ml. They were dialysed extensively, first against water and then against 25 m*M* triethanolamine–HCl pH 7.7. Protein solution (100–300 ml, corresponding to 15–20 mg of protein) was applied to a Mono Q column at a flow-rate of 2 ml/min. The elution gradients delivered by the pumps controlled by the programmer were different for each enzyme. They are shown in Figs. 4–6. The elution patterns shown in these figures demonstrate that HPIEC gives better resolution than conventional chromatography since for each of the three enzymes many contaminants, which eluted with enzyme activity from the DEAE-cellulose column, could be separated from the enzymatic protein on the HPLC column. This is also demonstrated by the analysis of the fractions by electrophoresis on polyacrylamide gels in the



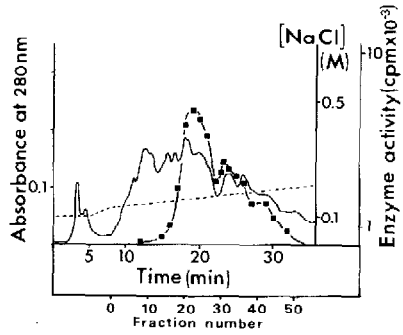


Fig. 4. HPIEC of O-methyltransferase I. Enzyme solution (100 ml) obtained from DEAE-cellulose chromatography (see Fig. 1) containing 15 mg of protein was injected onto the column at a flow-rate of 2 ml/min. The elution programme is illustrated by the change in sodium chloride concentration (----). Fractions of 0.5 ml were collected. The flow-rate was 1 ml/min. Absorbance was monitored (——) at 280 nm and the enzymatic activity of 10 μl of each fraction was assayed with 3 mM caffeic acid as substrate (■—■).

Fig. 5. HPIEC of O-methyltransferase II. Enzyme solution (90 ml) obtained from DEAE-cellulose chromatography (Fig. 1) containing 11 mg of protein was injected onto the column at a flow-rate of 2 ml/min. The elution programme is illustrated by the change in sodium chloride (----). Fractions of 0.5 ml were collected and the flow-rate was 1 ml/min. Absorbance was monitored (——) at 280 nm and the enzymatic activity of 20 μl of each fraction was assayed with 1.3 mM catechol as substrate (■—■).

presence of SDS. Fig. 7 shows a photograph of slab gel electrophoresis of chromatographic fractions containing O-methyltransferase II. Lane II shows the protein pattern of the peak of O-methyltransferase II obtained by DEAE-cellulose chromatography (Fig. 1), which was loaded on the HPLC column. Lanes 6 to 40 show the protein patterns of the corresponding fractions obtained by HPIEC illustrated in Fig. 5. The electrophoretical analysis of fractions 6–15 confirmed the presence of proteinaceous contaminants (see curve of absorbance at 280 nm of Fig. 5) that were eluted from the HPLC column before the active fractions containing O-methyltransferase II. These active fractions contained one major band (shown by the arrow) the intensity of which followed enzyme activity. Further purification (data not shown)

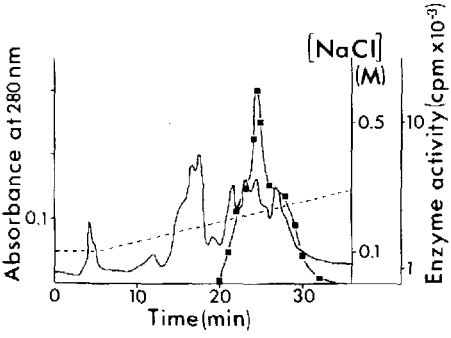


Fig. 6. HPIEC of O-methyltransferase III. Enzyme solution (250 ml) containing 15 mg of protein was injected onto the column. Other experimental conditions were as in Fig. 5. Elution programme (----), absorbance at 280 nm (——) and enzyme activity (■—■) are shown.

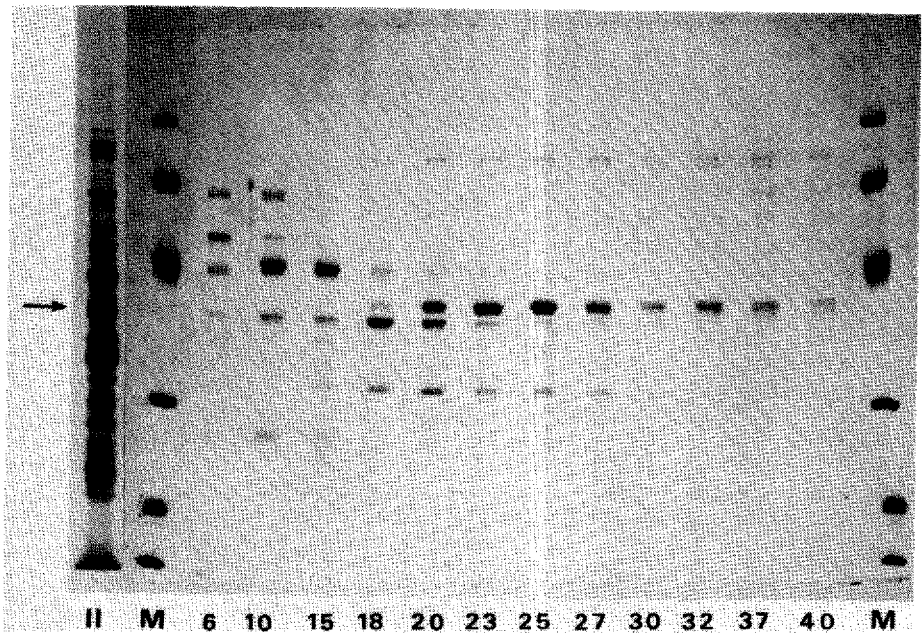


Fig. 7. Polyacrylamide gel electrophoresis of fractions obtained after HPIEC of O-methyltransferase II. Electrophoresis and staining of gels were carried out as described in Materials and Methods. Lane II shows the protein pattern of the peak containing O-methyltransferase II obtained after chromatography on DEAE-cellulose. This active fraction was used as such for loading the HPLC column but was concentrated before loading on the SDS slab gels. Lanes 6 to 40 show the protein patterns of the corresponding fractions obtained from the HPIEC illustrated in Fig. 5. Molecular-weight-marker proteins (M) were: phosphorylase *b* (MW 97,400), albumine (MW 67,000), ovalbumine (MW 43,000), carbonic anhydrase (MW 30,000), trypsin inhibitor (MW 20,100) and α-lactalbumin (MW 14,400).

suggests that this protein is likely to be a subunit of O-methyltransferase II. The comparison of the protein patterns of lane II with those of lanes 23 and 25, which correspond to the most active fractions, clearly demonstrates the progress in purification brought about by the HPLC step. Another important feature of HPLC on a preparative scale is the high rate of recovery of enzyme activity. The percentage recovery of activity ranged from 70 to 100% for the three enzymes.

In Fig. 4 the sharpness of the major peak indicates the high resolving power of the column but the peak of activity is rather broad and this suggests some heterogeneity in O-methyltransferase I. The same is observed for O-methyltransferases II and III (Figs. 5 and 6), enzyme II being eluted in two distinct peaks (Fig. 5). These activity profiles cannot have arisen from incomplete separation of the three enzymes after DEAE-cellulose chromatography since previous work showed that each O-methyltransferase was not contaminated by the others at this stage of purification[10]. The complexity of the activity profiles may be due to the presence of charge isomers of the enzymes but further investigations are needed to clarify this point.

Preliminary data show that contaminants which are still present in active fractions after HPIEC can be eliminated by other HPLC techniques, namely chromatofocusing and size-exclusion chromatography. We are now scaling up the purifica-

tion procedure aiming to raise specific antibodies towards tobacco O-methyltransferases.

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