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# Non-dialysis method of rapid and facile sample preparation for the desalting and purification of enzymes and other proteins from plant extracts

Moshe Shimoni* and Reuven Reuveni

*Division of Plant Pathology, Agricultural Research Organization, Newe-Ya'ar, Haifa 31999 (Israel)*

Michael Cais

*Department of Chemistry, Technion—Israel Institute of Technology, Technion City, Haifa 32000 (Israel)*

ABSTRACT

A self-contained sample preparation chromatographic device for the facile and rapid desalting, separation and purification of enzymes and other proteins is described. The method, dynamic column solid-phase extraction (DC-SPE), was applied to the extraction and separation of peroxidase isozymes from crude extracts of fresh leaf tissue of three maize lines and of cucumber leaf extracts, in screening plant resistance properties. When packed with Sephadex G-25(M), the DC-SPE device also gave highly satisfactory desalting of the plant protein solutions in a much shorter time (10–15 min) than that required to obtain similar results with Pharmacia DP-10 columns and/or by dialysis overnight.

## INTRODUCTION

The presence of multiple forms of peroxidase (EC 1.11.1.7) has been studied in connection with plant resistance [1–5], tissue specificity [6,7], induction of new cell wall biosynthesis [8,9], defence mechanism of plants in response to pathogen infection [8], cell wall injury [10] and disease resistance [11]. The cationic isozymes have been reported to constitute three quarters of the peroxidase activity in peanut cell medium [12] and they have been studied in relation to food flavours and food quality [13].

Dialysis and column gel filtration are commonly used sample preparation methods for removing low-molecular-mass compounds from solutions of macromolecules, prior to electrophoretic analysis. Dialysis of plant extracts is often carried out overnight at 4°C, with several buffer changes [14].

Dynamic column solid-phase extraction (DC-SPE) is a method for sample preparation that has been used for the extraction and analysis of food dyes [15], catecholamines, barbiturates and other analytes [16]. This paper describes the use of DC-SPE as an alternative to dialysis for sample preparation of plant leaf extracts and for the facile separation and purification of peroxidase cationic isozymes.

## EXPERIMENTAL

### *Materials*

Acrylamide, N,N-methylenebisacrylamide, ammonium peroxodisulphate, Coomassie Brilliant Blue R-250 and N,N,N′,N′-tetramethylenediamine were purchased from Bio-Rad Labs. Peroxidase type II (170 and 200 U/mg) and type X (275 U/mg), protein molecular mass markers

* Corresponding author.

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for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and benzidine hydrochloride were purchased from Sigma. DEAE-Sephacel and Sephadex G-25(M) were purchased from Pharmacia. Other materials were purchased from Merck, BDH and Frutarom Laboratory Chemicals (Israel).

The DC-SPE devices supplied by NBS New Biology Systems (Haifa, Israel) and by Frantz Medical (New York, U.S.A.), were made of medical grade polypropylene. The columns were packed in the collecting chamber of configuration A of the plunger column (Fig. 1) with 1.0 ml DEAE-Sephacel in 0.5 *M* Tris–HCl buffer (pH 7.5), or with 1.5 ml of Sephadex G-25(M) in the plunger column of configuration B (Fig. 1). Conditioning of the columns was carried out with $3 \times 2$ ml of 0.05 *M* Tris–HCl buffer (pH 6.8).

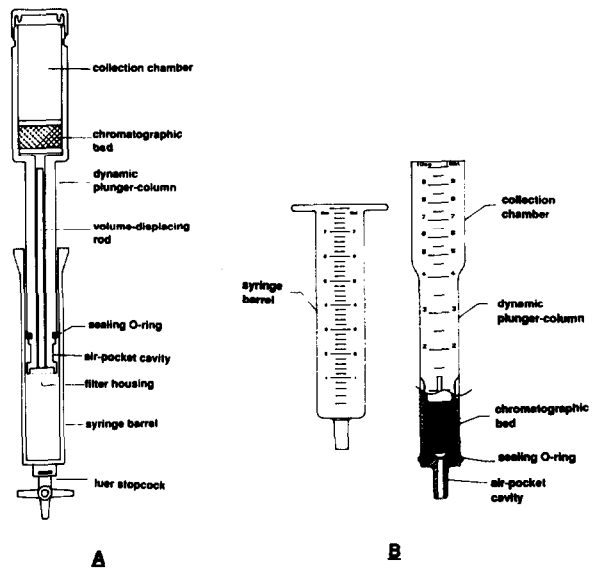


Fig. 1. Dynamic column solid-phase extraction device

*Dynamic column solid-phase extraction (DC-SPE)*

Recently, Cais *et al.* [17] reported the development of a concept in chromatography which they designated dynamic column liquid chromatography (DCLC). Briefly, in the DCLC concept, flow of the mobile phase is achieved by moving a packed chromatographic column through the eluent in a system that is completely closed, except for the column outlet. Under such conditions, an intrinsic pressure or vacuum develops in the system, which causes the mobile liquid phase to flow through the moving chromatographic bed in a direction opposite to the movement of the packed column.

In the DC-SPE modification [15], flow of mobile phase is achieved by moving a packed chromatographic plunger-column, fitted with a sealing O-ring, in and out of a syringe barrel with a Luer-locked stopcock fit. Fig. 1 shows two configurations, A and B, of the DC-SPE device. With the stopcock in the closed position, movement of the plunger column, downwards (PUSH-C) or upwards (PULL-C), causes an intrinsic pressure or vacuum, respectively, to develop in the system, forcing the mobile liquid phase to flow through the moving chromatographic bed in a direction opposite to the movement of the plunger column. With the stopcock in the open position, downward movement of the plunger column (PUSH-O) results in the expulsion of the liquid present in the syringe barrel; upward movement of the column (PULL-O) in this mode provides a means of aspirating liquid into

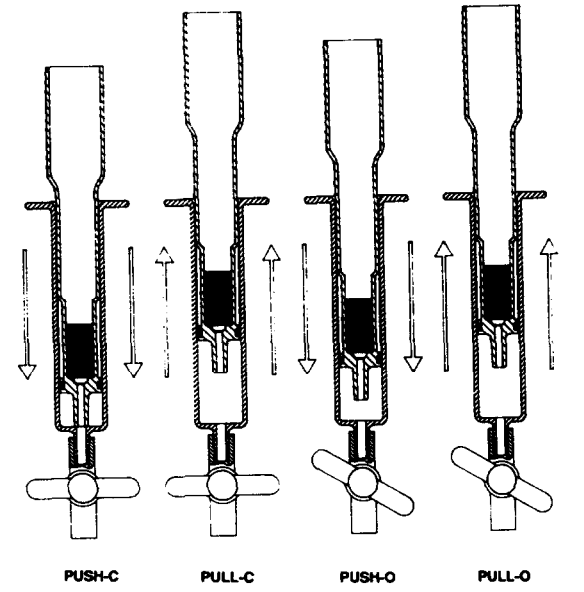


Fig. 2. Operational modes of the DC-SPE device. Stopcock closed: PUSH-C = downward plunger movement, PULL-C = upward plunger movement; stopcock open: PUSH-O = downward plunger movement, PULL-O = upward plunger movement.

the syringe barrel. The CLOSE (PULL-C and PUSH-C) and OPEN (PUSH-O and PULL-O) modes of operation of the DC-SPE device are shown schematically in Fig. 2.

*Preparation of enzyme extract*

Maize leaves were homogenized with a mortar and pestle in cold (4–6°C) 0.015 *M* sodium phosphate buffer (pH 6.0) or 0.05 *M* Tris–HCl buffer (pH 7.5). Cucumber leaves were homogenized, as above, in 0.05 *M* sodium acetate buffer (pH 5.0) or Tris–HCl buffer (pH 8.0) The homogenate was then centrifuged at 10 000 *g*. for 10 min (4°C). For maize leaves, the supernatant, without any additional work-up, was used as the crude extract in the purification procedures. For cucumber leaves, the supernatant was brought to 85% ammonium sulphate saturation and, after 1 h in an ice-bath, was centrifuged under the conditions as above. After discarding the supernatant, the precipitated pellet was dissolved in 0.05 *M* Tris–HCl buffer (pH 6.8) for further treatment.

*Peroxidase preparation and determination of activity*

Peroxidase (PO) type II and type X were diluted in 0.05 *M* sodium phosphate buffer (pH 7.0) to obtain concentrations of 0.2 mg/ml. PO activity was determined with 25 $\mu$l of sample using $H_2O_2$ and *o*-methoxyphenol (guaiacol) as described [4].

*Polyacrylamide gel electrophoresis (PAGE)*

Native or SDS discontinuous, non-denaturing polyacrylamide gels or slab mini gels were prepared according to Hames [18] and Davis [19] with a 2.5 or 4% stacking gel and 7.5, 10 or 12% separating gel. The resolving gel buffer was 0.375 *M* Tris–HCl (pH 8.8), the stacking gel buffer was 0.125 *M* Tris–HCl (pH 6.8) and the reservoir buffer consisted of 0.025 *M* Tris–0.192 *M* glycine (pH 8.8). Slab gels of 0.75 or 1.5 mm were prepared and run using the SE-600 apparatus (Hoefer Scientific Instruments) or the Mini Protean II apparatus (Bio-Rad Labs.) at 4–6°C.

*Gel staining and destaining*

SDS gels were rinsed with deionized water and transferred to a dilute Coomassie Brilliant Blue solution (0.001% Coomassie Brilliant Blue R-250 in 25% methanol–10% acetic acid). Bands appeared after 3–18 h without the need for destaining. Our double staining method for peroxidase and proteins in the same gel [20] was modified for use with aminoethylcarbazole or benzidine hydrochloride. Gels were soaked either in a solution containing 50 mg of benzidine hydrochloride in 50 ml of methanol and 150 ml sodium acetate buffer (0.05 *M*, pH 5.0) or in a solution made up of 30 mg of 3-amino-9-ethylcarbazole dissolved in 3 ml of N,N-dimethylformamide (DMF) and 150 ml of 0.05 *M* sodium acetate buffer (pH 5.0) containing 10 m*M* $H_2O_2$. After peroxidase staining, gels were restained with Coomassie Brilliant Blue by the same procedure as described above for SDS gels.

*Clean-up of peroxidase isozymes with configuration A (Fig. 1) DC-SPE columns*

Samples of 200 $\mu$l of maize leaf extracts or peroxidase standards (type II) were applied on top of the DC-SPE column and introduced into the DEAE-Sephacell bed by the PULL-C mode of operation and, after allowing the sample to equilibrate for 5 min on the chromatographic bed, the liquid (if any) in the syringe barrel was removed by the PUSH-O mode. A portion of 200 $\mu$l of buffer (0.05 *M* Tris–HCl, pH 7.5) was eluted through the column by sequential PULL-C and PUSH-O modes. The eluted fraction was discarded. Fractions of 400 $\mu$l were then eluted by the same procedure, with the same buffer, and collected. Each fraction was assayed for peroxidase activity.

*Sample preparation of cucumber leaf extracts with configuration B (Fig. 1) DC-SPE columns and comparison with dialysis*

A portion of the 0.05 *M* Tris–HCl buffer reconstituted solution (0.5 ml), after ammonium sulphate precipitation, was loaded on a DC-SPE plunger column packed with Sephadex G-25(M) by the PULL-C mode. On elution with Tris–HCl buffer, three fractions of 0.5 ml each were collected by sequential PULL-C and PUSH-O

modes, with a flow-rate time of 1 min per fraction.

A portion of the same reconstituted solution (0.5 ml) was applied to a Pharmacia PD-10 column packed with Sephadex G-25(M). On elution with Tris–HCl buffer, 15 fractions of 0.5 ml each were collected. The flow-rate was 2–3 min per fraction.

A portion of the same enzyme-extract reconstituted solution (1.0 ml) was dialysed overnight against two changes of 0.05 *M* Tris–HCl buffer (pH 6.8).

*Fractionation of peroxidase and other proteins from a plant extract with configuration B (Fig. 1) DC-SPE columns*

A portion (0.5 ml) of cucumber leaf extract [after dialysis with 0.05 *M* Tris–HCl buffer (pH 8.0)] was loaded on a DC-SPE column (packed with 1.5 ml of DEAE-Sephacel) by the PULL-C step. After allowing 5 min for equilibration, the peroxidase isozymes and other plant proteins were eluted by sequential PULL-C and PUSH-O steps with an NaCl gradient (0.0–1.0 *M*) in 0.05 *M* Tris–HCl buffer (pH 8.0). The collected fractions (1.0 ml each) were assayed for peroxidase activity and UV absorbance at 280 nm (see Fig. 6).

## RESULTS AND DISCUSSION

Classical sample clean-up methods often waste chemicals and time. The last few years have witnessed the introduction of a new approach for sample clean-up, solid phase extraction (SPE), in which the sample is passed, usually by application of suction, through a short bed of silica-based packing under conditions such as to retain completely the desired analytes. The column bed is then washed with an eluent to remove materials less strongly retained than the analytes and the desired analyte is then selectively eluted in a small volume of a stronger solvent.

The DC-SPE device described here is a complete, self-contained chromatographic unit designed for sample preparation and small-scale chromatography. There is no need for vacuum lines, pumps or centrifuges to generate flow. In addition, the principle of the DC-SPE device provides a bidirectional flow option to maximize concentration and elution of the sample. This option, not available in standard SPE tubes, offers the possibility of eluting analytes, which are often adsorbed at the top of the chromatographic bed, by the PUSH-C mode (Fig. 2), thereby removing them from the column without passing through the remainder of the chromatographic bed. This can result in smaller elution volumes and better total recoveries by diminishing losses through non-specific adsorption on the column. A closed system operation helps to prevent sample loss by accidental spillage.

The above properties and advantages of the DC-SPE device have previously been demonstrated in an application to the extraction and separation of synthetic food-colouring additives [15]. As, in general, ion-exchange chromatography for the separation of peroxidase isozymes from plant extracts [21–23] is time consuming and not suitable for the rapid screening of a large number of plant samples, we sought to overcome this problem by applying the DC-SPE methodology to the purification and separation of peroxidase isozymes.

Peroxidase type II and type X were used as standard markers for peroxidase activity. Standard protein markers containing bovine lactalbumin ($M_r$ 14 200), carbonic anhydrase ($M_r$ 29 000), chicken egg albumin, ($M_r$ 45 000) and bovine serum albumin monomer ($M_r$ 66 000) and dimer ($M_r$ 132 000) were also run. Several leaf extracts from maize (*Zea mays L.*) isolines B 37 (susceptible) and B 37 HtN (resistant) differing in resistance to *Exserohilum turcicum* and the susceptible sweet corn variety *Jubilee* were applied on top of the DC-SPE columns containing DEAE-Sephacel (as described under Experimental). Cationic peroxidase fractions, obtained in the flow-through, were analysed by PAGE along with the crude leaf extracts (from 6.6 mg of fresh leaf tissue) and peroxidase type II standards. Gels were double stained, first with benzidine and $H_2O_2$ and then with Coomassie Brilliant Blue R-250.

Fig. 3 presents a comparison of the electrophoresis of peroxidase isozymes of three corn strains, Jubilee (lanes a), B37 (lanes b) and B37 HtN (lanes c), before (lanes 1) and after (lanes

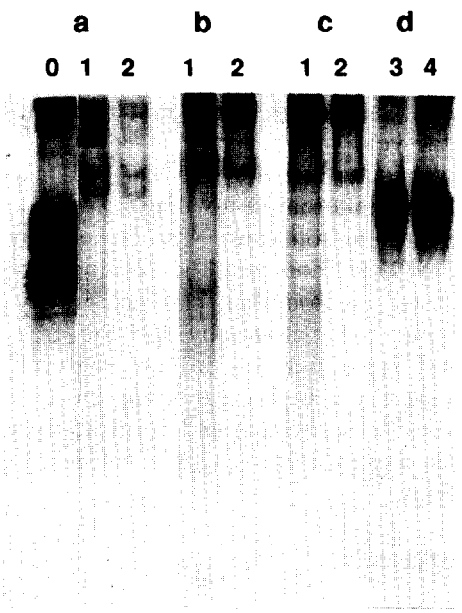


Fig. 3. Comparison of cationic peroxidase isozymes before (lanes 1) and after (lanes 2) separation by DC-SPE (on DEAE-Sephacel) from crude leaf extracts of three corn strains, Jubilee (lanes a), B37 (lanes b) and B37 HtN (lanes c). Peroxidase type II (as standard), before [lane 0 (a)] and after [lane 3 (d)] separation by DC-SPE is compared with peroxidase type X [in lane 4 (d)]. Staining of gels as described under Experimental.

2) separation by DC-SPE columns. The separation required less than 15 min. Peroxidase type II (as standard) is shown in lanes 0(a) and 3(d) before and after DC-SPE purification, respectively; lane 4(d) contains peroxidase type X standard. Lanes 0(a), 3(d) and 4(d) demonstrate the ability of DC-SPE to purify, in a simple and rapid operation, the lower grade type II peroxidase into the higher grade (and more expensive) type X peroxidase I fractions in the purification of type II peroxidase.

Fig. 4 presents the results of double staining, including peroxidase activity staining, of cucumber leaf proteins extracted by DC-SPE (lane 4) compared with extraction and purification by overnight dialysis (lane 2). The first fraction (0.5 ml) collected after the void volume (0.5 ml) with the DC-SPE device has a sufficient concentration of proteins to allow PAGE analysis and to provide results that are as good as those obtained in overnight dialysis.

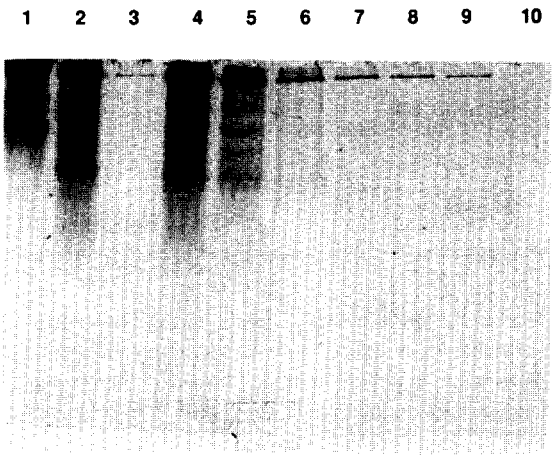


Fig. 4. Native PAGE of cucumber leaf proteins extracted as described under Experimental. Lane 1, peroxidase (EC 1.11.1.7) type II. Sample preparation for native electrophoresis by dialysis overnight (lane 2) or by DC-SPE, collecting eight fractions (0.5 ml each) (lanes 3–10); 10 $\mu$l of each fraction were loaded on 12% native polyacrylamide gel. Gel was double stained, first with aminoethylcarbazole and $H_2O_2$, followed by staining with Coomassie Brilliant Blue R-250.

Fig. 5a and b present electrophoresis data for comparison of three different sample preparation methods, namely overnight dialysis (lanes 2), DC-SPE (lanes 3) and Pharmacia PD-10 column gel filtration (lanes 4). Fig. 5a shows native electrophoresis data of peroxidase isozymes from cucumber leaf extracts, after double staining. Fig. 5b shows SDS-PAGE of the same samples as in Fig. 5a, but after protein staining with Coomassie Brilliant Blue R-250. Here again, it can be seen that the DC-SPE sample preparation results obtained in less than 15 min appear to be as good as those obtained by the more time-consuming methods. It is reasonable to assume that the rapidity of the DC-SPE sample preparation method should also provide better preservation of the enzyme activity, which is known to be adversely affected in long purification procedures.

An example of the separation of peroxidase enzymes and other proteins from cucumber leaf extracts using a DC-SPE column packed with DEAE-Sephacel (1.5 ml) and elution with an NaCl gradient (0.0–1.0 *M*) in 0.05 *M* Tris–HCl buffer (pH 8.0) is presented in Fig. 6. Peroxidase

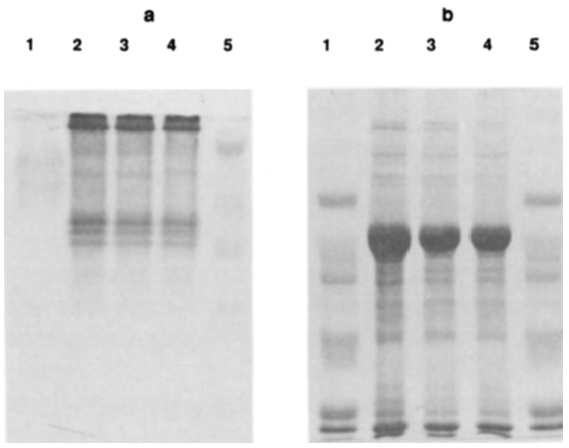


Fig. 5. (a) Separation of peroxidase isozymes from cucumber leaf extracts by three sample preparation techniques. Leaves of cucumber, infected with powdery mildew, were extracted as described under Experimental. Samples, after dialysis (lane 2), after DC-SPE (lane 3) and after PD-10 (Pharmacia) clean-up (lane 4), were loaded on native polyacrylamide gels. Lane 1 contains peroxidase (EC1.11.1.7) type II, lane 5 contains non-denatured protein markers (Sigma). Gels were stained, first for peroxidase activity (aminoethylcarbazole and $H_2O_2$), followed by Coomassie Brilliant Blue R-250. (b) SDS-PAGE of same cucumber leaf extracts as in (a). Lanes 1 and 5 contain molecular mass markers. Lane 2, sample after dialysis overnight; lane 3, sample after DC-SPE separation; lane 4, sample after clean-up on Pharmacia PD-10 columns. The gel was stained with Coomassie Brilliant blue R-250.

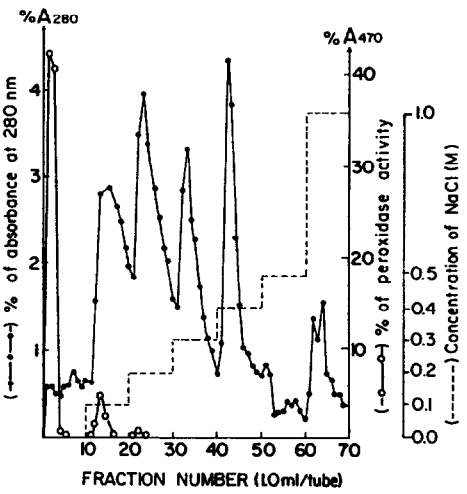


Fig. 6. Separation of peroxidase enzymes and other plant proteins by fractionation of a cucumber leaf extract on DC-SPE column (packed with 1.5 ml of DEAE-Sephacel). Elution (1.0-ml fractions) with an NaCl gradient (0.0–1.0 *M*) in 0.05 *M* Tris–HCl buffer (pH 8.0). Peroxidase activity and absorbance at 280 nm were measured for each fraction.

activity and absorbance at 280 nm were measured for each 1.0 ml fraction. As shown, the cationic peroxidase fraction (88 % of total peroxidase activity) was eluted in the first two fractions after the void volume.

The above results indicate that DC-SPE can be a satisfactory alternative to dialysis for the desalting of protein solutions. Also, it appears that the DC-SPE method could be developed to provide a faster method than classical gravity-elution column chromatography for the fractionation and separation of protein mixtures. Work in progress is aimed at extending the DC-SPE method to the separation and purification of other enzyme and protein systems of interest to plant pathology.

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