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PROCEDURE FOR SELECTION OF CELL WALL-ASSOCIATED GLYCOPROTEINS

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Key Word Index—*Nicotiana tabacum*; tobacco; cell wall enzymes; glycoprotein; peroxidase; laccase; extraction; purification.

Abstract—A novel, sequential extraction-affinity chromatographic procedure for the selection of high mannose-type glycoproteins from the ionically bound proteins of plant cell walls is reported. Ionically bound proteins were extracted using a modified binding buffer for Concanavalin-A, the extract passed through Concanavalin-A Sepharose and high-mannose type glycoproteins selectively desorbed using α-methyl mannoside. Extraction with the ConA-binding buffer compared favourably with previously used extraction methods in terms of the purity of peroxidase and the range of peroxidase isozymes recovered from tobacco cell walls. The sequential affinity chromatography step cleanly selected peroxidase activity and provided a significant purification *via* a reduction in bulk protein. This procedure has great potential as a convenient and robust, initial step in the purification of a wide range of cell wall-associated high-mannose type glycoproteins.

INTRODUCTION

The use of molar solutions of mono-, di- or trivalent salts to extract ionically bound proteins from crude preparations of plant cell walls (detailed in ref. [1]) has become widely accepted and applied routinely [2, 3], but has certain disadvantages. Firstly, it may yield inaccurate information because intracellular and membrane proteins may become artificially associated with the cell wall during homogenisation [4]. Secondly, as efficient extraction requires reasonable volume to weight ratios, large volumes of concentrated salt solutions must be handled and desalted prior to further purification. Desalting and subsequent concentration steps are usually time-consuming and can influence the yield and/or the activity of the enzyme under study. The present work illustrates the use of a novel procedure for extraction and purification of cell wall-associated glycoproteins to extract peroxidase from tobacco cell walls.

RESULTS AND DISCUSSION

The results of a representative experiment are given in Table 1. The use of M NaCl extracted three times more protein and 1.5-fold more peroxidase activity from crude tobacco cell walls than the use of the ConA buffer (50 mM Tris–HCl pH 7, containing 100 mM $CaCl_2$ and 1 mM $MnCl_2$), but at half the specific activity. In addition, the lengthy procedure required to obtain the concentrated desalted sample resulted in no net purification and a considerable (60%) loss in peroxidase yield. In comparison, the affinity chromatography step produced the bound fraction (Fig. 1) at a small net purification with a 50% loss in peroxidase yield. However, the separation of the unbound peroxidase activity (see Fig. 1) must be taken into account when considering this loss of yield, as this unbound peroxidase, unlike the peroxidase activity lost from the M NaCl procedure, can be collected and examined.

It is also important to note that the same set of anionic and cationic peroxidase isozymes are extracted by the two methods (Fig. 2). Differences in the relative abundance (e.g., the cationic isozymes appear to be less abundant in the M NaCl extraction than the ConA extraction) may be explained as equal amounts of protein, not activity, were applied. Analysis of the extracts by SDS-PAGE revealed that the ConA method results in a cleaner, more distinct banding of proteins (Fig. 3, cf. lanes B and D), which is advantageous for purification.

Extraction with the ConA buffer compared favourably with the M NaCl extraction in terms of the purity of peroxidase and the range of peroxidase isozymes recovered. Combined with the Concanavalin-A affinity chromatography step, the overall method provides a rapid initial purification and may prove to

Table 1. Comparison of extraction methods

Sample	Specific activity (nmols ABTS min^{-1} μg^{-1})	Total activity (nmols ABTS min^{-1})	Volume (mL)	Protein (μg)
NaCl extract	37.1 ± 0.6*	172 533	180	4644
Concentrated desalt	35.5 ± 0.9	68 987	30	1941
ConA extract	76.1 ± 0.9	108 698	170	1428
Bound fraction	87.3 ± 2.7	57 653	30	660

* These values are averages of three replicates ± standard error.

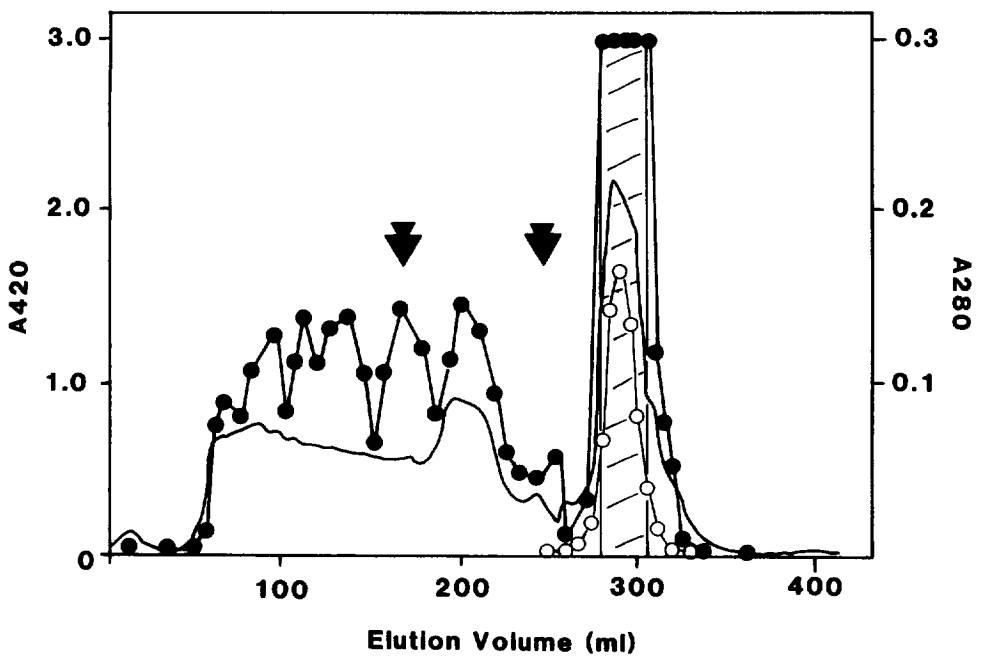

Fig. 1. Recovery of cell-wall associated peroxidases by affinity chromatography on Concanavalin-A Sepharose. Details are given in the Experimental. Filled symbols = peroxidase activity of fractions (10 μl samples, 15 min assay); empty symbols = peroxidase activity of fractions (2 μl samples, 10 min assay). Solid line = A_{280} of fractions. First arrow marks the start of the wash with ConA buffer and the second arrow marks the start of elution with ConA buffer plus 100 mM α-methyl mannoside. The hatched area was pooled as the bound fraction.

be a useful step in the purification of glycoproteins associated with the plant cell wall, such as the laccase-type [5, 6] and non-laccase-type [7, 8] polyphenol oxidases implicated in lignification, invertases [9] and other high-mannose type glycoproteins of, as yet, unknown function [10]. It is, of course, essential that before this method is used for other cell-wall-associated glycoproteins that the efficiency of extraction and the affinity for the lectin is checked. Initial studies have shown that ConA buffer, when vacuum-infiltrated into tobacco stems, will efficiently extract peroxidase activity, which can be enriched using a micro-version of the affinity chromatography technique. This adaptation may have particular value in monitoring alterations in the amounts of cell wall-associated enzymes in response to stresses, such as wounding [11] or environmental pollution [12].

The technique has some disadvantages. Ubiquitous cell wall glycoproteins such as peroxidase [13], can swamp other glycoproteins but this is also true of the previously used methods. Mn^{2+} ions, which are required for efficient binding of Concanavalin-A to glycosyl groups [14], may interfere with the activity of enzymes, especially metalloenzymes. However, they can be omitted from the extraction and binding buffers without significant effect on yield or recovery (results not shown) if the lectin is suitably loaded with Mn^{2+} ions prior to use. More seriously, leaching of Concanavalin-A from affinity matrices has been reported [15]; this extraneous glycoprotein may complicate further purification.

EXPERIMENTAL

Plant tissue and extraction of cell wall associated proteins. Tobacco plants (*Nicotiana tabacum* cv. Samsun) were grown as described previously [16]. Plants were harvested 8 weeks after planting, the third to

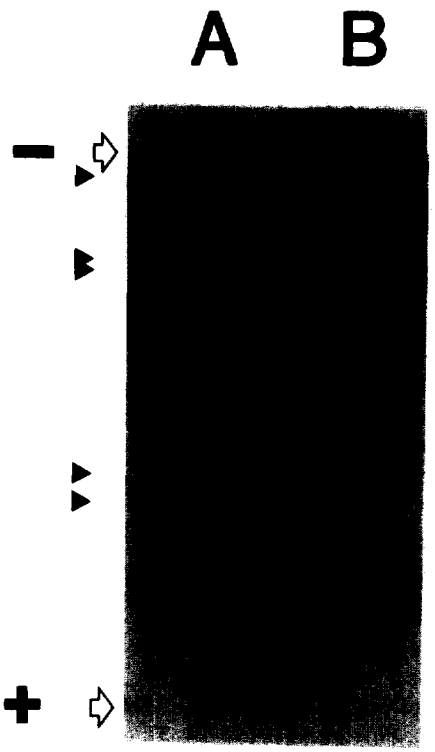

Fig. 2. Separation of peroxidase isozymes by isoelectric focusing. The position of the cathode and anode are marked. The relative mobility of the marker proteins is marked by arrow points (p*I*s from cationic to anionic are 9.6, 7.5, 6.8, 4.7 and 4.5). Lane A contains 2 μg protein from the ConA-bound fraction and lane B contains 2 μg protein from the concentrated desalted sample.

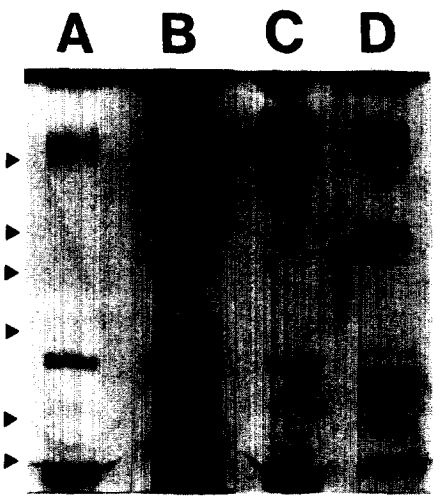

Fig. 3. SDS-PAGE of tobacco cell wall extracts. Each lane contains 1 μg protein. Lane A = 1 M NaCl extract; lane B = concentrated desalted; lane C = ConA extract; lane D = ConA-bound fraction. Positions of M_r proteins (97 400, 66 200, 45 000, 31 000, 21 500 and 14 400) are shown.

sixth internodes excised and the stem cut longitudinally down to the xylem using a razor blade. The tissues external to the xylem (i.e. the phloem, parenchyma and epidermis) were peeled off, cut into 5 mm pieces across the direction of growth and stored on ice. Exactly 100 g of this tissue was homogenised in excess ice-cold 50 mM Tris–HCl pH 7.5 using a Waring blender (5 × 60 sec bursts, full power, on ice) then an Ultra-Turrax disintegrator (5 × 30 sec bursts, full power, on ice). After filtration through glass sinters, the residue was resuspended in fr. buffer and the procedure repeated × 4. At this point, the crude cell wall residue was weighed and split in half. One half was extracted in 170 ml 50 mM Tris–HCl, pH 7 containing M NaCl, the other half in 170 ml 50 mM Tris–HCl, pH 7 containing 100 mM $CaCl_2$ and 1 mM $MnCl_2$ (ConA buffer) for 1 hr on ice. Extracts were collected by centrifugation at 2500 g for 10 min at 8°.

Dialysis and concentration. The M NaCl extract was desalted by extensive dialysis against 5 mM Tris–HCl pH 7 (3 × 5 l changes, at 8° over 16 hr). The dialysate was concd by reverse dialysis against dry polyethylene glycol compound (Sigma product no. P2263) at 8° until the desired vol. was reached.

Procedure for Concanavalin-A Sepharose. A 50 ml bed vol. column of Concanavalin-A Sepharose (Pharmacia) was pre-equilibrated in cold ConA buffer at 1 ml min^{-1}. Buffers for affinity chromatography on Concanavalin-A Sepharose require Ca^{2+} and Mn^{2+} ions (usually 1 mM) to ensure efficient binding of the lectin [14] and usually contain 100 mM NaCl to prevent non-specific binding of proteins to the matrix [15]. The use of 100 mM $CaCl_2$ serves to prevent non-specific binding and provides Ca^{2+} ions for lectin-binding. The ConA cell wall extract (150 ml) was loaded onto the column, followed by a wash of 80 ml of ConA buffer then 160 ml of ConA buffer containing 100 mM α-methyl mannoside. Frs (5 ml) were collected and assayed for peroxidase activity and A_{280}. No further peroxidase activity could be eluted from the column using M NaCl.

Assays for peroxidase activity and protein content. Peroxidase activity was measured by monitoring the increase in A_{420} due to the formation of the oxidised chromophore of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as described previously [7]. Protein content was measured by the dye-binding method [16].

Isoelectric focusing and SDS-PAGE. Isoelectric focusing (p*I* range 3–10) was carried out using a Mini-IEF system according to the manufacturer's instructions (Bio-Rad). Where required, protein solns were concd using centrifugal membrane concentrators (Centricon-10 units, Amicon) and desalted using PD-10 columns (Pharmacia). Samples (up to 2 μg protein) were loaded onto the surface of the gel and IEF marker proteins (Bio-Rad) were run as a guide to the pI of the focused bands. Peroxidase activity was detected by incubating the gels in 3.64 mM ABTS in 100 mM NaOAc pH 5 containing 2.5 mM H_2O_2 for 10 min and photographs taken immediately. Analytical SDS-PAGE was carried out using a Mini-Protean II slab gel system according to the manufacturer's

instructions (Bio-Rad) Standard low M_r range marker proteins were run and gels stained using a silver-staining kit (Bio-Rad).

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