Phytochemistry, 1979, Vol. 18, pp. 1049–1054. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0601-1049 $02.00/0

ADSORBENT POLYSTYRENE AS AN AID IN PLANT ENZYME ISOLATION

W. DAVID LOOMIS, JACK D. LILE, RICHARD P. SANDSTROM and ALICE J. BURBOTT
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, U.S.A.

(*Received* 20 *September* 1978)

Key Word Index—Plant enzyme isolation; polystyrene resins; Amberlite XAD-4; PVPP; Polyclar AT; plant phenolics; isothiocyanates; terpenes.

Abstract—Porous polystyrene (Amberlite XAD-4) adsorbs hydrophobic and surface-active materials from plant extracts and homogenates. With conventional extraction techniques, these materials tend to bind to proteins by hydrophobic interactions. In some cases covalent modification of protein also occurs. Interfering compounds include phenolics, quinones, terpenes and organic isothiocyanates. Polystyrene complements insoluble polyvinylpyrrolidone (PVPP, Polyclar AT), and the combination of these two adsorbents produced superior enzyme extracts from the several plant tissues that were tested. Tested procedures are described, and suggestions are given for adapting the procedures to new plant systems with contaminating natural products varying in quantity and in chemical nature.

INTRODUCTION

Plants produce a large variety and frequently large quantities of secondary products [1–3]. These cause special problems in the isolation of enzymes and organelles [4–6], as well as nucleic acids [6a, 6b], from plant tissues. Many of these secondary products bind tightly to proteins and are not removed by classical isolation procedures. Both covalent and non-covalent complexes are formed between secondary products and proteins. We previously described the use of insoluble polyvinylpyrrolidone (polyvinylpolypyrrolidone, PVPP, Polyclar AT) to remove those plant phenolic compounds which form strong H-bonded complexes with proteins [4–6], but some phenolic compounds, as well as certain other interfering secondary products, are not bound effectively by PVPP.

Hydrophobic binding of low molecular weight organic compounds to proteins in aqueous media has been studied by several investigators. Mohammadzadeh *et al.* [7, 8] found that the binding varies with the structures of both components. Binding of straight-chain alkanes was greatest in the range from C_5 to C_9. Ester groups or C=C double bonds enhanced the binding and promoted the binding of larger hydrophobic molecules to proteins. Benzene was bound in particularly large amounts. The molecular sizes and polarities of these strongly-bound hydrophobic compounds are similar to many of the plant secondary products, such as terpenes, phenolic monomers, and organic isothiocyanates.

Lam and Shaw [9] reported that the polystyrene-based anion exchange resin, Dowex 1-X8, at pH 8.3, removed phenolics from extracts of flax (*Linum usitatissimum* L.) cotyledons. Recently, Gray [10] reported that Dowex 50, a sulfonated polystyrene cation exchange resin, also binds plant phenolics effectively (at pH 7.5). Lam and Shaw pointed out that Dowex 1 might bind anionic proteins, while Gray did not consider possible binding of proteins by the ion exchange resins. Earlier research on protein fractionation [11, pp. 396–401] makes it clear that several ion exchange resins, both anion exchangers and cation exchangers, can bind proteins. Much of the adsorptive and fractionating capacity of polystyrene-based ion exchange resins is due to hydrophobic binding by the polystyrene matrix [12]. Polystyrene resins cross-linked with divinylbenzene are now widely used to concentrate, and to fractionate low molecular weight organic compounds from aqueous solutions [13–15]. They have also been used to remove detergent from plant enzyme extracts [16].

We previously reported [17] that treatment of enzyme extracts from mint with porous polystyrene beads (Amberlite XAD-4) removed contaminating monoterpenes, which resisted separation by other procedures including gel filtration. We have also briefly described the use of polystyrene resins to remove secondary products, including phenolics, from plant enzyme extracts in a review on plant enzyme methods [6]. Plant enzyme extraction methods have also been reviewed recently by Rhodes [17a]. We wish here to describe the use of adsorbent polystyrene in more detail and to present new information on purification and use of the resins.

RESULTS AND DISCUSSION

Comparisons of adsorbents

Several polymeric adsorbents were tested for their effectiveness in removing representative phenolics from aqueous solutions. Typical results are shown in Table 1. The values shown are corrected for dilution by the H_2O contained in the hydrated polymers. We have subsequently discovered (see below) that UV-absorbing materials may leach from the resins even after extensive washing. Thus the binding capacity of the resins may be somewhat greater than the data indicate. The phenolic materials tested were selected as model systems. Tyrosine is ubiquitous in living organisms. It serves as a substrate for plant phenolases [18], and the resulting quinones react readily with proteins [4]. Chlorogenic acid and quercetin are widespread plant phenolic compounds [1] and potential quinone precursors. The quinone formed

Table 1. Percent decrease in UV absorbance* of model phenolic systems after treatment with phenol adsorbents

Phenolic system†	Adsorbent				
	Polyclar AT	Dowex 1-X8	Dowex 2-X8	Amberlite XAD-4	Amberlite XAD-7
Tyrosine, 0.4 mM	13	9	23	22	17
Chlorogenic acid, 0.1 mM	76	97	100	60	56
Quercetin, 0.1 mM	82	91	100	83	86
Masonex (California), 0.02%‡	50	80	81	72	57
Masonex (Mississippi), 0.02%‡	62	68	81	57	51

* 20 ml of test solution treated with 4 g of adsorbent (dry wt of Polyclar AT, hydrated wt for the other adsorbents). UV absorbance measured at 274 nm for tyrosine, 324 nm for chlorogenic acid, 365 nm for quercetin and 270 nm for Masonex.

† Dissolved in 0.1 M potassium phosphate buffer, pH 6.5.

‡ Based on wet weight as received. 0.012% based on reported dry matter content.

by enzymatic oxidation of chlorogenic acid has been shown to add covalently to protein functional groups [19]. Semiquinone radicals are probably involved as well [20].

'Masonex' is a mixture of water-soluble wood extractives produced as a by-product in the manufacture of 'Masonite' hardboard from wood fiber. It is produced in California, largely from redwood [*Sequoia sempervirens* (Lamb.) Endl.], and in Mississippi, from a mixture of softwood and hardwood species (Dale Galloway, Masonite Corp., Chicago, personal communication). The two types of Masonex differ somewhat in their analysis, but both contain primarily polysaccharides and phenolics and are 60% dry matter [21]. Masonex undergoes base-catalysed oxidation in the presence of air. The pH curve of O_2 uptake suggests oxidation of ionized phenolic residues to quinones (Lile, J. D. and Loomis, W. D., unpublished).

With the exception of tyrosine, all of the model phenolics were more or less effectively bound by each of the adsorbents. The poor adsorption of tyrosine implies that it does not form the strong non-covalent complexes that many plant phenolics do, and that conventional procedures such as gel-filtration would remove it from extracts.

The various adsorbents tested bind phenolics in different ways. PVPP contains —CO—N< groups that are analogous to the peptide bonds of proteins. This group is a strong proton acceptor and forms stable H-bonded complexes with phenolic —OH groups [4–6]. Both Dowex resins contain quaternary ammonium groups on a styrene-divinylbenzene matrix [Dowex 1: ϕ-$CH_2N^+(CH_3)_3$; Dowex 2: ϕ-$CH_2N^+(CH_3)_2(C_2H_4OH)$]. They bind by a combination of ionic and hydrophobic interactions, with probably some H-bonding in the case of Dowex 2.

Amberlite XAD-4 and XAD-7 are porous beads made from synthetic resins. XAD-4 is a styrene-divinylbenzene polymer with a surface area of 725 $m^2\ g^{-1}$ and an average pore diameter of 40 Å. Binding is primarily by hydrophobic interactions [22, 23]. Nominally it has no ion exchange capacity, but we observe a slight change in color in alkaline media, suggesting the presence of ionizable groups. Amberlite XAD-7 is an acrylic ester polymer with a surface area of 450 $m^2\ g^{-1}$ and an average pore diameter of 90 Å. It is somewhat polar, and is capable of both polar and hydrophobic binding [24].

Amberlite XAD-2 (cross-linked polystyrene) is a similar product to XAD-4, but with less total surface area (300 $m^2\ g^{-1}$) and larger average pore diameter (90 Å) [22, 25]. Amberlite XAD-4 was used in this study because of its high surface area. We tested Amberlite XAD-2, in comparison with XAD-4, for capacity to adsorb catechol from a 0.1 mM solution at pH 6.8, and it bound considerably less than did XAD-4. However, we did not test it further. According to the manufacturer [22], the interaction between surface area and porosity is such that Amberlite XAD-4 has a much higher capacity than XAD-2 for small molecules such as phenol, while for larger molecules such as alkylbenzene sulfonates, Amberlite XAD-2 is more effective than XAD-4.

The manufacturers report that Amberlite XAD-7 binds some protein [24], and that proteins can be eluted from the resin with salt solutions. They suggest that the resin may be useful in concentration or fractionation of proteins [24]. However, the possible binding of proteins made it seem less desirable than the polystyrene resins for routine use in removing secondary products from enzyme extracts, and it was not tested further. If additional testing of polyester resins is to be done, Amberlite XAD-8 with its much larger pore size should also be considered (average pore diameter 225 Å: surface area 160 $m^2\ g^{-1}$) [22].

Our results with the Dowex ion exchange resins confirm the reports of Lam and Shaw [9] and Gray [10] of the ability of Dowex 1 to bind plant phenolic compounds. These results also indicate that the high pH's used by Lam and Shaw (pH 8.3) and by Gray (pH 7.5) are not necessary. The reported ineffectiveness of Polyclar AT in removing plant phenolics at pH 8.3 [9] is not surprising, since the binding by PVPP depends on the presence of non-ionized phenolic hydroxyl groups [4–6]. High pH is in general undesirable in the extraction of plant enzymes because it increases the ionization of phenolic hydroxyl groups and thus promotes oxidation of phenols, with subsequent protein modification [6, 20]. Gregory and Bendall [26] reported that in assays of tea leaf polyphenol oxidase, non-enzymatic blanks (base-catalysed oxidation of the *o*-dihydroxy phenolic substrates) were high at pH's above 6.0, and prohibitively high above pH 6.6. High pH also increases the proportion of protein amino groups in the reactive —NH_2 form, making them more susceptible to addition reactions. Optimum results in the isolation of plant enzymes and organelles are generally obtained by extracting at a pH of 6.0–7.2 [6].

Tests of PVPP and polystyrene in combination

The effectiveness of polystyrene-based ion exchange resins in removing phenolic materials is undoubtedly due to a combination of polar and hydrophobic binding. The polar interactions may include hydrogen bonding as well as ion exchange [27]. PVPP and polystyrene used together provide a similar combination, though the polar binding in this case is presumed to involve only hydrogen bonding. We have tested the combination of Polyclar AT and Amberlite XAD-4 qualitatively in the preparation of extracts from several plant tissues. The tissues were ground in liquid N_2 with a mortar and pestle, and mixed into a slurry of the two adsorbents in H_2O (1 g dry wt of Polyclar AT and 1 g hydrated wt of Amberlite XAD-4 per g fresh tissue, plus H_2O to make a thick slurry). After filtration, the resulting extracts were compared on the basis of soluble protein content (turbidity with 5% trichloroacetic acid and the presence of a UV absorption peak at 280 nm), and the absence of other UV and visible absorption—notably the absence of browning. Effective removal of phenolic materials should eliminate browning and reduce the UV absorption at 260 nm. It will also reduce the absorption at 280 nm but should uncover an absorption peak at that wavelength due to protein.

The combination of Polyclar AT and Amberlite XAD-4 produced superior extracts even from refractory plant tissues. Tissues tested included potato tubers, apple fruit, peppermint leaves, English walnut hulls and black walnut hulls. All of these plant extracts contained soluble protein, in contrast to the frequent lack of soluble proteins in conventional extracts from plants [4–6]; and qualitative assays for several enzymes (e.g. alkaline phosphatase) were positive. Black walnut hulls were the least promising of the tissues tested. While all of the other tissues gave colorless extracts, extracts from black walnut hulls were pale yellow, even with increased amounts of Amberlite XAD-4. However, these walnut extracts contained soluble protein and had high alkaline phosphatase activity, and the pale yellow color was in sharp contrast to the usual brown-black color of walnut homogenates and extracts. Walnut tissues contain a glucoside precursor of juglone, a quinone noted for its reactivity [3]. Column treatment of peppermint extracts with Amberlite XAD-4, after initial extraction in the presence of Polyclar AT, removes persistent monoterpene contaminants and makes it possible to study terpene metabolism in cell-free systems by direct GC analysis, whereas ^{14}C-substrates were required previously ([17] and Burbott, A. J. and Loomis, W. D., unpublished).

Removal of organic isothiocyanates

Further tests of the adsorbents were carried out with horseradish roots [28]. These results will be described in detail elsewhere [29], but observations regarding the use of the adsorbents are relevant here. Horseradish roots are a rich source of organic isothiocyanates [30]. Isothiocyanates are very reactive [31], and they react readily with proteins [32]. Variability in the isozyme patterns of horseradish peroxidase (HRP) has been reported [33], suggesting possible protein modification.

Initially, a H_2O extract of the roots was prepared with a juice extractor, and portions of it were treated batchwise with Polyclar AT (0.4 g dry wt/12 g tissue) and Amberlite XAD-4 (0.8 g hydrated wt/12 g tissue), both singly and in combination. The untreated extract had a sharp, pungent odor of isothiocyanates, and a dark yellowish color. Amberlite XAD-4 removed most of the odor, while each of the adsorbents contributed to removal of visible color. The combination of adsorbents removed all of the visible color. UV absorbance was still high, but a distinct shoulder could be seen at 403 nm (Soret band of heme). Increasing the amounts of adsorbents, especially of Amberlite XAD-4, reduced the overall UV absorbance and revealed a distinct protein peak at 280 nm.

Subsequently, extracts were prepared by homogenization in a Waring Blendor in the presence of H_2O and Amberlite XAD-4, followed by filtration with a basket centrifuge, and further treatment with both adsorbents, and then concentrated by precipitation with ammonium sulfate. These extracts were entirely free of isothiocyanate odor if enough Amberlite XAD-4 was used. Ammonium sulfate precipitation proceeded normally in the adsorbent-treated extracts. In untreated extracts, or in extracts that had not been adequately treated, the precipitates floated, suggesting the formation of a lipid–protein complex. Our extraction procedure, except for the use of adsorbents, and the additional H_2O needed to accommodate them, was as described by Maehly [34] for isolation of HRP. Maehly also noted [34] that the ammonium sulfate precipitate floated.

In spite of these indications that adsorbent treatment of horseradish extracts removed contaminants, isoelectric focusing of the peroxidases gave variable results, even with as much as 115 g of hydrated Amberlite XAD-4 per 100 g of tissue present during homogenization. Sometimes the odor of horseradish oil was detected during extraction, and more Amberlite XAD-4 had to be added. These results convinced us that the reaction of the isothiocyanates with horseradish proteins is extremely rapid, and that the initial stages of homogenization and extraction are crucial. Better extraction, and consistent isoelectric focusing patterns, are now obtained by pulverizing the tissue in liquid N_2 (Janke and Kunkel laboratory mill, from Tekmar Co., Cincinnati, Ohio) and thoroughly mixing the frozen powder with 2.5 to 4 times its weight of hydrated Amberlite XAD-4 in an ice bath, with no additional water or buffer. (The hydrated polymer contains about 53% polymer and 47% H_2O by weight.) Water is then mixed in slowly to produce a thick slurry, which is filtered and given additional adsorbent treatments as before. The amounts of Amberlite XAD-4 used here include a large safety factor, but a safety factor is essential if reactive secondary products are present. As an additional safety factor, a small amount of Amberlite XAD-4 is added to the extracts at every stage of the purification procedures. The effectiveness of this extraction procedure appears to depend largely on the extremely fine pulverization of the tissue that is obtained by grinding in liquid N_2 and the lack of chemical reactions at this low temperature, plus the close contact between adsorbent and plant material that is obtained by mixing them in the absence of fluid water.

Tests of possible protein binding

To check for possible adsorption of proteins by Amberlite XAD-4, tests were carried out with horse-

radish peroxidase and bovine serum albumin (BSA). A solution of 1 mg HRP/ml, in H_2O, (measured pH of the solution was 4.0), was shaken with 50 mg of the hydrated resin per mg HRP, and filtered. The A_{403} decreased slightly, in agreement with the expected dilution from the approximately 47% of H_2O in the hydrated resin. The A_{275} increased slightly, but the same increase was observed in pure H_2O shaken with the resin. The same commercial HRP (50 mg in 5 ml H_2O) was passed through a 1 × 20 cm column of Amberlite XAD-4 and analysed by isoelectric focusing. This treatment produced no change in either the positions or the intensities of peroxidase bands. BSA (1 mg/ml, in H_2O) was treated with hydrated Amberlite XAD-4 which had been shaken with H_2O and blotted dry immediately before use (25 mg of resin/mg BSA). In this case the A_{280} decreased only by the approximately 1% expected from dilution.

It appeared from our trials that neither of the test proteins was adsorbed by the polystyrene resin. BSA is a water soluble protein which functions as a lipid carrier [6, 35]. The fact that it is not bound by Amberlite XAD-4 makes it seem unlikely that any protein that is physiologically water soluble would be bound. At the same time, Schechter and Bloch [16] have removed detergent (deoxycholate) from solubilized plant membrane proteins with Amberlite XAD-2. It seems probable that even hydrophobic membrane proteins would not interact strongly with the adsorbent polystyrene, because the 3-dimensional structures of both protein and resin would hinder contact between extensive surface areas of the two. However, a warning is in order. At low pH (5–5.8 or below) there appears to be non-specific binding of many proteins to ion exchange resins [11, p. 381; 36]. Boardman and Partridge [36] suggested that this binding was due to H-bonding by the —COOH groups of their polyacrylic resin. Fasold *et al.* [11] argued that the binding must be due to partial unfolding of proteins and interaction with the hydrophobic matrix of their sulfonated polystyrene resin. It seems reasonable to suggest that in both cases there was a combination of hydrophobic interactions and H-bonding, with protein —COOH groups playing an important role. If the hydrophobic binding of proteins to the resin matrix is strong, one would expect adsorbent polystyrene to behave similarly to the ion exchange resins. Our findings suggest that HRP, at least, does not bind hydrophobically to Amberlite XAD-4 at low pH. HRP is stable at low pH, and the heme group is readily removed from the protein by extraction with methyl ethyl ketone at pH 2 [37]. HRP adjusted to pH 2 with HCl was treated with Amberlite XAD-4 as described above. In this case the A_{403} was 10% less than expected, and the A_{275} was 3% more than expected. The effects of low pH on the absorption spectrum, and possibly on extraction of contaminants from the resin, make quantitative interpretation uncertain, but one can conclude that there was little, if any, binding of either HRP protein or heme.

General applications

With all of the plant tissues we have tested, Amberlite XAD-4 and Polyclar AT complement each other, but the relative contributions of the two adsorbents vary from one tissue to another. For example, Polyclar AT removes very little of the browning-precursor from potato tubers, while Amberlite XAD-4 removes nearly all of it. With peppermint leaves and apple fruit tissue, exactly the opposite is true. In the case of peppermint, Amberlite XAD-4 removes a small amount of highly reactive material that is not removed by Polyclar AT, and which if not removed makes protein fractionation virtually impossible. The Amberlite resin also removes contaminating monoterpenes from peppermint extracts [17]. With horseradish roots, rapid removal of isothiocyanates with Amberlite XAD-4 is of critical importance, but Polyclar AT does remove a certain amount of colored material. Gross *et al.* [38] reported that Polyclar AT and Dowex 1 complemented each other in a similar way in isolating enzymes from *Taxus* leaves.

Because plant tissues vary so widely in their content of secondary products, no universal prescription for enzyme extraction procedures can be given. Each investigator must, by testing, determine the required amounts of adsorbents and the most effective way of applying them to his particular plant system. Very often one or the other of the adsorbents will be needed in large amounts. As a guide, we use 1 g dry wt (*ca* 5 g hydrated wt) of Polyclar AT per g of fresh peppermint leaves, and we are currently using even higher levels of Amberlite XAD-4 (1 g dry wt = *ca* 2 g hydrated wt) in extracting horseradish roots.

Polyclar AT can be used either pre-hydrated or dry. Thorough hydration increases the capacity for adsorbing phenolic compounds by about 50% [4]. On the other hand, dry PVPP can be used as a convenient agent for concentrating extracts. One g dry wt of Polyclar AT yields about 4.8 g hydrated wt. Homogenization of Polyclar AT causes partial degradation, with production of soluble PVP [4]. We have recommended [4] that Polyclar AT should not be present during tissue homogenisation, as this may result in contamination of enzyme extracts by soluble PVP-phenolic complexes. If Polyclar AT is to be stored or used in the dry form, we recommend that it be broken into chunks before drying, and that no effort be made to reduce these chunks to powder. The chunks hydrate readily, and pulverization is likely to produce soluble PVP as a contaminant. Polyclar AT is very convenient for batchwise treatment of homogenates, but columns have slow flow rates.

Amberlite XAD-4 can be used in a variety of ways. Columns pack easily and have rapid flow rates. Batchwise addition of the hydrated polymer during tissue homogenization (e.g. Waring Blendor) provides rapid removal of hydrophobic secondary products. However, we have recently determined that even after extensive washing with acetone, dilute aqueous HCl, and H_2O, the polymer bleeds UV-absorbing materials into H_2O (peak absorbance at 260 nm). Homogenization in H_2O in a Waring Blendor increases the rate of release of UV-absorbing materials. There is no indication that these materials react with protein, but users should be aware of the possible complications, e.g. in spectrophotometric analyses. Ion exchange resins are well known to give similar problems. The principal contaminants in the Amberlite polystyrene resins are reported to be naphthalene, ethylbenzene, and benzoic acid [14]. Of these, benzoic acid seems most likely to bleed into H_2O.

Use of finely-ground Amberlite resins rather than the intact beads might provide improved mixing and contact in batchwise treatments. A procedure which appears very promising for horseradish, but which needs further

testing, is the addition of finely-ground dry Amberlite XAD-4 to the powdered plant material in liquid N_2. Thorough mixing results. After the mixture has warmed to 0°, it is held in an ice bath for several minutes to allow enzymatic release of isothiocyanates, before adding H_2O to extract proteins. The volatile isothiocyanates apparently diffuse into the gas-filled pores of the resin and are adsorbed there. There is no detectable odor of isothiocyanates at any stage of the procedure.

Removal of the adsorbents in batchwise treatment can be accomplished rapidly by squeezing through bolting silk or by filtering in a basket centrifuge. A commercial juice extractor with the cutter plate removed provides an effective and inexpensive basket centrifuge. The Model No. 6001 Juicerator produced by the Acme Juicer Mfg. Co., Sierra Madre, California, is heavier duty than those generally available in retail stores, and has given us good service. The centrifuge basket is lined with Miracloth or filter paper cut to fit. Glass fiber filter paper (Whatman GF/B) provides a superior combination of high speed and high retentiveness compared to cellulose filter papers.

Purification of polystyrene resins

Purification of the Amberlite resins before use is important. They are technical grade polymers, supplied in hydrated form, with NaCl and Na_2CO_3 as preservatives [23–25], as well as various organic impurities. The manufacturer [23] recommends backwashing with H_2O on a column for about 10 min, or until classification of the beads by size is achieved. This is followed in sequence by about 5 bed volumes of MeOH and about 20 bed volumes of H_2O passed downflow through the column.

We tested several solvents for possible use in purifying Amberlite XAD-4. These included MeOH and absolute EtOH (both alone and with 1% of conc HCl added), acetone and dimethylformamide. All of these appeared about equally effective. Batchwise washing and column treatment both appeared to be effective. For reasons of economy as well as convenience we now use Soxhlet extraction with acetone, as described in the Experimental.

Junk *et al.* [14] have used the Amberlite polystyrene resins in columns to concentrate trace contaminants from H_2O. Thorough purification of the resins is essential for their use. They tested a variety of purification procedures, and they now extract sequentially with MeOH, acetonitrile, and ether, for 8 hr each in a Soxhlet extractor, using more than one cycle of the solvents if necessary. The beads are then stored under MeOH, and washed in the column with H_2O just before use. This procedure cleans the adsorbent surface, but apparently occluded organic impurities are still present and are readily released after any physical disruption of the beads —even by stirring to remove air bubbles, if it is not done very carefully, or by drying (Junk, G. A., personal communication).

EXPERIMENTAL

Materials. Polyclar AT from GAF Corporation, New York, N.Y., was acid-washed as described previously [6]. Dowex 1-X8 (50–100 mesh), and Dowex 2-X8 (20–50 mesh) were washed with 1 N HCl, followed by MeOH containing 1% of conc HCl, until no UV-absorbing (260 nm) material was detectable in the eluate. Amberlite XAD resins were from Rohm and Haas Co., Philadelphia, Pennsylvania. Before use they were suspended several times in distilled H_2O and the fines decanted. They were then washed with a polar organic solvent until no more colored material was eluted, then twice with 3 N HCl, followed by glass-distilled H_2O until the washings gave a negative Cl^- test ($AgNO_3$) and zero absorbance at 260 nm. For the tests described in Table 1, the resins were washed in a column with MeOH. For reasons of convenience and economy, we now wash Amberlite XAD-4 with Me_2CO in a Soxhlet extractor, changing the Me_2CO after 1–2 extraction cycles and extracting for several days, or ca 25 cycles. Acid and H_2O washing are as before, either by batchwise washes with stirring, or by backwashing. Downflow column washing without stirring is much less efficient. The resin is stored under H_2O and given a final H_2O wash just before use. If the resin is to be used dry rather than hydrated, it is washed as described, then oven-dried, ground in a Wiley mill, washed several times with MeOH, and air-dried. When the beads are used in a column, they can be regenerated by backwashing with MeOH followed by H_2O.

L-Tyrosine was obtained from Mann Research Laboratories. Chlorogenic acid was J. T. Baker Chemical Co. 'Baker Grade'. Quercetin was a purified crystalline sample prepared from Douglas fir bark [*Pseudotsuga menziesii* (Mirb.) Franco]. It was supplied by Prof. E. F. Kurth of Oregon State University. Samples of Masonex were supplied by Mr. Dale Galloway of the Masonite Corporation, Chicago, Illinois. Other chemicals were reagent grade. Glass-distilled H_2O was used throughout unless otherwise specified. Commercial HRP was Worthington code HPOD, $A_{403}/A_{275} = 1.1$. BSA was Calbiochem C Grade.

Plant materials. Potato tubers (*Solanum tuberosum* L., cv Russet Burbank) and apple fruit (*Pyrus malus* L., cv delicious) were purchased in local markets. Peppermint (*Mentha piperita* L., cv Black Mitcham) was grown in the greenhouse, propagated vegetatively from the same clone that we have used previously. Walnut hulls (*Juglans regia* L., English walnut, and *Juglans nigra* L., black walnut) were picked green from local trees. Horseradish roots [*Armoracia rusticana* (Gaertn.) Mey. et Scherb.] were field grown. Those used in the initial studies in Palmerston North were obtained from a local grower, through Prepared Foods Limited. Those used in Corvallis were cv Maliner Kren, propagated from roots obtained from the Burpee Seed Co., Riverside, California.

Analytical methods. Protein in plant extracts was determined by the trichloracetic acid turbidity method [39, 6]. Purified proteins (BSA, HRP) used in testing of methods were assayed by UV absorption [34, 40]. Thin-layer isoelectric focusing was done essentially as described by Delincée and Radola [41], using 1.2% Brinkmann pH 2–11 'pHisolytes' plus 0.1% arginine and 0.1% lysine. Peroxidase bands were detected by the print technique of Delincée and Radola, with *o*-dianisidine [41]. Alkaline photophatase was assayed by the colorimetric method of ref. [42].

Adsorption of phenolics. For the tests described in Table 1, chlorogenic acid and quercetin were dissolved in 0.1 M potassium phosphate buffer, pH 6.5, to give a concn of 0.1 mM. Masonex was diluted with the same buffer to a concn of 0.02% Masonex, or 0.012% dry matter [21]. 20 ml of each soln was stirred with 4 g of adsorbent for 2 min and filtered through Whatman No. 1 filter paper. (Polyclar AT was 4 g dry wt and was then hydrated before use. The other adsorbents were 4 g hydrated wt.) Absorption spectra of the filtrates were measured from 210 to 800 nm. The decrease in absorbance after treatment (corrected for H_2O in the adsorbents) is reported for each model system at the wavelength of maximum absorbance. The conditions employed in other tests are described with the results.

Acknowledgements—This research was supported by grants from the National Science Foundation, the U.S. Brewers Associa-

tion, the Masonite Corporation, the Oregon State University Environmental Health Sciences Center and the Oregon State University Research Council. Part of the work was carried out during the tenure of an N.Z.R.A.C. Senior Research Fellowship by W. D. L., in the Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand. We thank Mr. Louis Blecher of GAF Corporation, New York, N.Y., and Mr. John Bossler of Rohm and Haas Co., Hayward, California, for advice and for samples of adsorbents.

REFERENCES

1. Robinson, T. (1975) *The Organic Constituents of Higher Plants*, 3rd edn. Cordus Press, P.O. Box 587, North Amherst, Massachusetts.
2. Karrer, W. (1958) *Konstitution und Vorkommen der organischen Pflanzenstoffe (exclusive Alkaloide)*. Birkhäuser, Basel.
3. Thomson, R. H. (1971) *Naturally Occurring Quinones*, 2nd edn. Academic Press, London.
4. Loomis, W. D. and Battaile, J. (1966) *Phytochemistry* **5**, 423.
5. Loomis, W. D. (1969) in *Methods in Enzymology* (Lowenstein, J. M., ed.) Vol. 13, p. 555. Academic Press. New York.
6. Loomis, W. D. (1974) in *Methods in Enzymology* (Fleischer, S. and Packer, L., eds.) Vol. 31, p. 528. Academic Press, New York.

6a. Smillie, R. M. and Krotkov, G. (1960) *Can. J. Botany* **38**, 31.

6b. Newbury, H. J. and Possingham, J. V. (1977) *Plant Physiol.* **60**, 543.

7. Mohammadzadeh-K., A., Feeney, R. E. and Smith, L. M. (1969) *Biochim. Biophys. Acta* **194**, 246.
8. Mohammadzadeh-K., A., Smith, L. M. and Feeney, R. E. (1969) *Biochim. Biophys. Acta* **194**, 256.
9. Lam, T. H. and Shaw, M. (1970) *Biochem. Biophys. Res. Commun.* **39**, 965.
10. Gray, J. C. (1978) *Phytochemistry* **17**, 495.
11. Fasold, H., Gundlach, G. and Turba, F. (1961) in *Chromatography* (Heftmann, E., ed.) p. 378. Reinhold, New York.
12. Moore, S. and Stein, W. H. (1951) *J. Biol. Chem.* **192**, 663.
13. Grieser, M. D. and Pietrzyk, D. J. (1973) *Analyt. Chem.* **45**, 1348.
14. Junk, G. A., Richard, J. J., Grieser, M. D., Witiak, D., Witiak, J. L., Arguello, M. D., Vick, R., Svec, H. J., Fritz, J. S. and Calder, G. V. (1974) *J. Chromatogr.* **99**, 745.
15. Still, G. G. and Mansager, E. R. (1975) *Pestic. Biochem. Physiol.* **5**, 515.
16. Shechter, I. and Bloch, K. (1971) *J. Biol. Chem.* **246**, 7690.
17. Croteau, R., Burbott, A. J. and Loomis, W. D. (1973) *Biochem. Biophys. Res. Commun.* **50**, 1006.

17a. Rhodes, M. J. C. (1977) in *Regulation of Enzyme Synthesis and Activity in Higher Plants* (Smith, H., ed.) p. 245. Academic Press, London.

18. Baruah, P. and Swain, T. (1959) *J. Sci. Food Agric.* **10**, 125.
19. Pierpoint, W. S. (1969) *Biochem. J.* **112**, 609 and 619.
20. Synge, R. L. M. (1975) *Qual. Plant.* **24**, 337.
21. Masonite Corporation (1975) *Product Sheets, Masonex 225*. Sheets No. 5429 (Mississippi) and 5430 (California). Masonite, Chicago.
22. Rohm and Haas Company (1978) *Summary Bulletin, Amberlite Polymeric Adsorbents*. Rohm and Haas, Philadelphia.
23. Rohm and Haas Company (1978) *Amberlite XAD-4*. Rohm and Haas, Philadelphia.
24. Rohm and Haas Company (1969) *Preliminary Technical Notes, Amberlite XAD-7*. Rohm and Haas, Philadelphia.
25. Rohm and Haas Company (1975) *Amberlite XAD-2*. Rohm and Haas, Philadelphia.
26. Gregory, R. P. F. and Bendall, D. S. (1966) *Biochem. J.* **101**, 569.
27. Olsson, L., Renne, N. and Samuelson, O. (1976) *J. Chromatogr.* **123**, 355.
28. Loomis, W. D., Battaile, J., Burbott, A. J., Stout, H. A. and Pearce, P. (1977) *Plant Physiol.* **59** (suppl.), 7.
29. Loomis, W. D. *et al.*, in preparation.
30. Gilbert, J. and Nursten, H. E. (1972) *J. Sci. Food Agric.* **23**, 527.
31. Kawakishi, S. and Namiki, M. (1969) *Agric. Biol. Chem.* **33**, 452.
32. Stark, G. R. (1970) in *Advances in Protein Chemistry* (Anfinsen, C. B., Jr., Edsall, J. T. and Richards, F. M., eds.) Vol. 24, p. 261. Academic Press, New York.
33. Liu, E. H. and Lamport, D. T. A. (1973) *Arch. Biochem. Biophys.* **158**, 822.
34. Maehly, A. C. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 2, p. 801. Academic Press, New York.
35. Foster, J. F. (1960) in *The Plasma Proteins* (Putnam, F. W., ed.) Vol. 1, p. 179. Academic Press, New York.
36. Boardman, N. K. and Partridge, S. M. (1955) *Biochem. J.* **59**, 543.
37. Teale, F. W. J. (1959) *Biochim. Biophys. Acta* **35**, 543.
38. Gross, G. G., Mansell, R. L. and Zenk, M. H. (1975) *Biochem. Physiol. Pflanz.* **168**, 41.
39. Kunitz, M. (1952) *J. Gen. Physiol.* **35**, 423.
40. Layne, E. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 3, p. 447. Academic Press, New York.
41. Delincée, H. and Radola, B. J. (1972) *Analyt. Biochem.* **48**, 536.
42. Worthington Biochemical Corp. (1977) *Worthington Enzyme Manual* (Decker, L. A., ed.) p. 138. Worthington, Freehold, New Jersey.
43. Nieman, R. H., Pap, D. L. and Clark, R. A. (1978) *J. Chromatogr.* **161**, 137.

NOTE ADDED IN PROOF

Nieman *et al.* [43] have recently published a method for the purification of plant nucleotides using Amberlite XAD-2, PVPP and charcoal to remove interfering phenolics.