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ANALYSIS OF CELL WALL MATERIAL FROM PLANT TISSUES: EXTRACTION AND PURIFICATION

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Abstract—The alcohol-insoluble residue (AIR) of immature and mature runner beans contains co-precipitated cytoplasmic proteins, nucleic acids, starch and polyphenols, which contaminate the isolated polysaccharide fractions and their binding is sufficiently tenacious to resist complete extraction with the usual protein solubilizing reagents. Therefore, a method was developed for preparation of "cell wall material" from plant tissues in which the contamination with cytoplasmic constituents was minimal. Alternative solvents for cell disruption and protein extraction have been compared. The method depended for its success on the selective removal of the contaminants from fresh ball-milled tissue by sequential treatments with 1% aq. Na deoxycholate, PhOH–HOAc–H_2O followed by α-amylase digestion. Ball-milling the tissue ensured almost complete rupture of the cells and organelles and allowed the solvents to penetrate the sample fully and dissolve the cytoplasmic constituents. The purified "cell wall material" has protein contents varying from 2·5 to 5·5% depending on the type and maturity of the tissue. The residual proteins are resistant to pronase, rich in hydroxyproline and have the amino acid composition of purified cell wall proteins, showing that the wall preparations are relatively pure.

INTRODUCTION

Most of the methods available for the fractionation and isolation of the various cell wall constituents from plant tissues use either the alcohol (or Me_2CO) insoluble residue (AIR) [1–6] or the alcohol–C_6H_6 extractive free residue [7] as the starting material. While useful information about cell wall constituents could be obtained from such residues, their inherent disadvantages have often been overlooked. For example, most of the cytoplasmic proteins (from membranes, enzymes, etc.), nucleic acids and reserve polysaccharides like starch are co-precipitated with the residues in addition to some of the low molecular weight compounds like polyphenols and phosphate esters. Adsorption of soluble proteins by cell wall fragments has been discussed by Newcomb [8] and the factors affecting the interaction of polyphenols and their oxidation products (quinones) with proteins, thus impairing their solubility, have been reviewed by Loomis and Battaile [9], while Cherry and Chroboczek [10] and Isherwood and Barrett [11] have discussed the factors affecting nucleic acid and phosphate ester extractability from plant tissues respectively. The cytoplasmic protein contamination of the AIR could vary from 5–40% of its weight depending on the type and maturity of the tissue [4, 12]. This contamination is quite considerable compared with the cell wall protein content of the tissue, which is about 2–6% by weight of the cell wall material. In the case of tissues rich in polyphenols (e.g. tea leaves and bark of trees), an appreciable quantity of the polyphenols contained in the vacuoles are co-precipitated with the membrane proteins [3]. The co-precipitated polyphenols, proteins, nucleic acids and starch considerably alter the relative weights of the various groups of cell wall polysaccharides and tend to obscure completely the significance and type of cell wall proteins associated with them. Further, in the

case of tissue containing large quantities of starch (e.g. potatoes [13] and tea roots [3]), the starch is not completely extracted by the aqueous solvents used for the sequential removal of polysaccharides from AIR and the solubilized and residual starch interfere with the isolation of cell wall constituents. Therefore, it seems clear that at least a preliminary partial fractionation resulting in the complete removal of these contaminants is desirable.

In the present paper, the whole extraction procedure has been considered in detail as a necessary preliminary to the final analysis of the cell-wall preparation. Various aqueous solvents and procedures have been tried in an attempt to remove the cytoplasmic proteins and other contaminants and their relative merits considered. A method which provides a cell wall preparation virtually free from these contaminants is described.

RESULTS

Preliminary experiments with alcohol-insoluble residue (AIR). The results of preliminary experiments in which the AIR of immature and mature beans were fractionated into the various groups of polysaccharides, their percentage protein content (most of which is of cytoplasmic origin as shown by amino acid analysis) and the sugars liberated from them on acid hydrolysis are given in Table 1. The protein content of the pectin extracted residue increased by 7–10% and this increase is reflected in the relatively high levels of protein in the holocellulose and hemicellulose A. Very few proteins were associated with the α-cellulose fraction. Ribose was detected in the hydrolysates of the "pectic fraction" and it could possibly have arisen from co-precipitated nucleic acids. Ribose was also detected in the hydrolysates of the pectic fraction from tea shoots [4]. Several sugars were detected in the "polymers" isolated from the chlorite soluble fraction.

Attempts to remove the co-precipitated proteins from the AIR with various protein solubilizing reagents and pronase were only partially successful. Sequential extraction of the AIR of immature and mature beans and parenchyma of mature beans (a tissue devoid of lignin) with deoxycholate and phenol–acetic acid–water removed only about 30–40% of the co-precipitated proteins showing that lignin (of mature tissues) is not the primary component responsible for their incomplete removal. Prolonged treatment (6 days) of the AIR with pronase in Tris buffer removed about 90% of the co-precipitated proteins and some cell wall polysaccharides (mainly pectic substances). It was found

Table 1. Percentage composition of the alcohol-insoluble residue of immature and mature runner beans

Constituent	Immature beans		Mature beans		Sugars detected in hydrolysates
AIR		(25·0)		(16·0)	
$CHCl_3$–MeOH soluble compounds	0·68		0·16		
Pectic substances	38·8	(4·8)	41·8	(3·8)	Galacturonic acid, galactose, arabinose, glucose, rhamnose, xylose, ribose
Hexametaphosphate extracted AIR		(36)		(22·5)	
$NaClO_2$-soluble compounds (lignin, adsorbed polyphenols and some proteins)	18·2		9·6		Galactose, arabinose, xylose, glucose, uronic acid
Holocellulose	41·7	(32·6)	48·5	(16·6)	
Hemicellulose-A	6·0	(24·1)	12·0	(14·3)	Xylose, glucose, galactose, arabinose
Hemicellulose-B	1·4		0·8		Glucose, xylose, mannose, galactose, arabinose, uronic acid
α-Cellulose	31·9	(2·2)	34·1	(1·4)	Glucose, galactose, arabinose, uronic acid

The figures in parentheses are the percentage protein content of the polysaccharide fractions. For each fraction, the sugars liberated on hydrolysis are listed in approximately descending order of concentration. Values for $NaClO_2$ soluble compounds were obtained from calculated difference of hexametaphosphate extracted residue before and after the delignification step. While the pectic substances have been corrected for coprecipitated hexametaphosphate, the hemicelluloses have not been corrected for coprecipitated KOAc.

that the AIR became "more fibrous" on removal of co-precipitated proteins (and compounds adsorbed by proteins).

Extraction of fresh plant tissues with different solvents. In order to test the effectiveness of the various solvents for solubilizing cytoplasmic constituents, immature (slightly lignified) and mature (heavily lignified) runner bean pods and asparagus shoots (a material containing both slightly and heavily lignified tissues) were used. Several solvents, all of which have been used for the isolation of either cell wall material or cytoplasmic proteins from plant tissues, were tried. The solvents were 80% aq. EtOH (at 70°) [1], distilled H_2O [14], 1·5% (w/v) aq. sodium dodecyl sulphate (SDS) [15], 1% (w/v) aq. sodium deoxycholate (SDC) [16], PhOH–HOAc–H_2O (PAW) [17] (2:1:1, w/v/v, the water content of the tissue being taken into consideration) and 1% SDC followed by PAW. The use of salt solutions was avoided as this tended to result in the solubilization of appreciable quantities of pectic substances as well. For the last 5 methods of extraction, the temperature was between 0 and 5°. Since the last procedure, i.e. sequential extraction of the ball-milled tissue with SDC and PAW, gave the most complete extraction of cytoplasmic proteins, nucleic acids and polyphenols, this is described in the form finally developed for the isolation of cell wall material in a state of purity. A similar procedure was used with the other solvents. Steaming the tissue (to inactivate the enzymes) prior to extraction with SDC did not appreciably alter the protein level of the final residue or its amino acid composition.

Extraction with SDC and PAW (preferred method). The plant material (50 g, usually sliced) was frozen in liquid N_2 ground to a powder and then blended with 1% SDC (100 ml) for 5 min, octanol (~2 ml) being added as an anti-foaming agent. Thin tissues like immature beans and leaves could be blended directly. The macerate was transferred with 100 ml 1% SDC to a Pascall ball-mill container (1 litre internal vol.) and ball-milled for 15–20 hr at 1°. In the case of tissues (e.g. potatoes) which tend to go dark brown on ball-milling, it is preferable to incorporate sodium metabisulphite (3 mM) in the extraction medium. With most tissues no intact cells could be detected by light microscopic examination after the procedure. The ball-milling time was dependent on the type and maturity of the tissue, heavily lignified tissues requiring longer times. The ball-milled suspension was filtered using a nylon sieve and the balls washed with distilled water to remove any adhering material. The suspension was then centrifuged at 15000 *g* for 10 min at 1°. The supernatant was decanted and the scum adhering to the sides of the centrifuge tube was carefully removed. The residue was washed twice with three bed volumes of distilled H_2O on the centrifuge. The wet residue (vol. 5–10 ml) was then extracted twice with 25 ml PAW (a short treatment in a blender helped to give a uniform suspension). The centrifuged residue was then washed with H_2O, alcohol and Et_2O on the centrifuge and dried under reduced pressure over silica gel. The final residue was found to be a reasonably pure cell wall preparation for tissues containing little or no starch. In the case of tissues containing an appreciable quantity of starch (e.g. potatoes), the preparation was de-starched by treatment with α-amylase as below.

Removal of starch from cell wall material. Starch was removed by treatment with α-amylase at 30° for 36 hr [18]. The quantity of α-amylase required depended on the starch content of the tissue and no difficulty was experienced in removing the bulk of the starch from mature potatoes. In the preliminary experiments the starch was removed from the final residue (200 mg) of potatoes by incubation with α-amylase (0·2 mg) (activity: 500–1000 units/mg). In later experiments it was realized that it would be easier to remove the starch by incubating the cholate extracted residue with α-amylase. The residual amylase was removed by washing with 0·02 M phosphate buffer on the centrifuge before proceeding with the PAW extraction. The absence of starch in the cell wall preparation was confirmed by a negative reaction in the I_2/KI test. The above procedure did not result in any significant loss of cell wall constituents because very dilute neutral phosphate buffer (0·02 M) was used.

Effectiveness of different solvents. In this study the protein content of the cell wall preparation, the resistance of the residual proteins to pronase action and its hydroxyproline level are used as an index of purity of the wall preparation. The results given in Table 2 show the effect of different solvents on the yield of crude cell wall material from runner beans and asparagus and their percentage protein content. The weights of the insoluble resi-

Table 2. The effect of ball-milling treatment and solvent used on the yield of "cell-wall material" from immature and mature runner beans and asparagus

Solvent (vol. used, ml)	Yield of "cell-wall material" from 100 g fresh weight of tissue			
	Immature beans	Mature beans	Parenchyma†	Asparagus‡
80% Aq. alcohol (400)	5·15 (25·0)	5·40 (16·0)	1·81 (24·4)	2·76 (16·8)
Cold H_2O (400)	3·81 (18·7)	4·30 (7·7)	1·55 (12·0)	2·40 (8·0)
1% SDC (400)	3·36 (7·4)	4·44 (4·8)	1·42 (7·8)	2·29 (4·5)
1·5% SDS (400)	3·38 (6·6)	4·38 (6·9)		
PAW* (500)	3·35 (6·1)	4·22 (3·6)	1·30 (5·4)	2·05 (5·0)
1% SDC (400) and PAW (100)	3·30 (5·4)	4·25 (2·7)	1·10 (4·8)	2·00 (3·5)

The figures in parentheses are the percentage protein content of the "cell-wall material". * The PAW macerate was not ball-milled because of difficulties in handling it. † Parenchyma from mature beans—the tissue was obtained by scraping the opened pods with a spoon up to the parchment layer. ‡ The lower 5 cm of asparagus shoots 15–20 cm long.

dues with the various aq. solvents were comparable and were considerably less than that of the AIR. Although a small quantity of pectic substances is solubilized by the aq. solvents, these results, together with those for different tissues of the tea plant [3, 4], tend to reflect the amount of co-precipitation not only of proteins, but also of other low molecular weight compounds which occur during extraction with alcohol. Distilled water proved to be quite inefficient, even with non-lignified tissues, although it has been used for the preparation of hydroxyproline rich cell wall material from cell suspension cultures [14, 19]. SDS and SDC proved to be equally efficient for solubilizing the bulk of the cytoplasmic proteins. PAW was generally better. However, sequential extraction with SDC and PAW gave wall preparations containing the least amount of proteins. The residual proteins could not be solubilized with pronase. For routine analysis SDC was the preferred first solvent because maceration with SDS resulted in excessive foaming.

Table 3 shows the amino acid compositions of the AIR from immature and mature beans, SDC soluble proteins from immature and mature beans and asparagus, SDC/PAW soluble proteins from mature beans and that of the final insoluble residues from all three tissues. The composition of the SDC soluble proteins is comparable with that of the AIR because the latter contains most of the co-precipitated proteins. From Tables 2 and 3 it is clear that while the bulk of the cytoplasmic proteins could be removed with either SDS or SDC, further treatment of the residue with PAW resulted

Table 3. Amino acid composition (mol/100 mol of amino acids) of proteins from immature and mature beans and asparagus

Amino acid	Immature beans			Mature beans			Asparagus†	
	AIR	SDC soluble from fresh beans	SDC/PAW insoluble from fresh beans	AIR	SDC soluble from fresh beans	SDC/PAW* soluble from fresh beans	SDC/PAW insoluble from fresh beans	SDC soluble from fresh shoots	SDC/PAW insoluble from fresh shoots
Hydroxyproline	2·9	1·3	14·3	3·7	0·8	3·5	22·3	0·8	10·8
Aspartic acid	10·9	12·8	6·6	10·7	11·1	11·0	5·9	17·2	6·2
Threonine	5·2	5·1	4·3	5·2	5·5	5·8	3·3	5·0	4·5
Serine	7·8	8·0	8·3	7·4	7·3	9·4	6·9	7·6	8·4
Glutamic acid	10·9	11·6	8·2	11·0	11·8	8·9	6·8	11·5	8·3
Proline	6·0	5·7	6·7	6·3	5·3	8·0	9·4	5·3	6·0
Glycine	9·0	8·3	9·9	8·1	9·0	10·6	7·3	8·2	12·9
Alanine	9·1	9·7	5·6	9·1	9·6	8·6	4·3	9·4	9·2
½ Cystine	0·4	0·2		0·3	2·9	0·2		0·7	0·4
Valine	5·3	4·8	6·2	5·8	5·8	6·7	6·5	5·1	4·9
Methionine	1·0	0·5	0·6	0·4	1·3		0·9	0·5	1·0
Isoleucine	3·4	3·1	3·6	3·6	3·8	3·4	3·0	3·3	2·7
Leucine	8·8	9·2	6·3	9·0	9·5	7·8	5·1	8·1	7·4
Tyrosine	2·8	2·7	3·9	2·8	2·4	3·3	4·0	2·2	2·4
Phenylalanine	3·7	4·2	2·7	3·8	4·2	3·9	2·3	3·5	3·3
Lysine	7·3	6·9	7·2	7·1	6·9	4·1	7·2	6·3	6·7
Histidine	1·6	1·5	2·8	1·9	1·6	1·8	2·6	1·3	2·2
Arginine	3·9	4·4	2·8	3·8	4·2	3·3	2·2	4·0	3·7

* Proteins solubilized by PAW from SDC extracted material. † The lower 5 cm of asparagus shoots 15–20 cm long.

in the extraction of a protein, the amino acid composition of which is more comparable with that of the SDC soluble proteins than cell wall proteins. The PAW solubilized protein appears to be more firmly bound to the cell wall constituents and may be involved in their metabolism. The amino acid compositions of the final residues (SDC/PAW insoluble) are comparable with those of wall proteins from other plant tissues [14, 19–21], a characteristic feature being the relatively high levels of hydroxyproline and proline. The hydroxyproline content of the cell walls of mature beans is considerably more than that of immature beans and asparagus. These results, in addition, give some idea of the composition of the wall proteins from dicotyledon and monocotyledon tissues.

Hydrolysis (2 N H_2SO_4 for 10 hr) of the precipitates (obtained on addition of EtOH) from the SDC soluble extracts from immature and mature beans gave glucose, galactose, arabinose, galacturonic acid and a little ribose. These sugars could have arisen from cold water soluble polysaccharides, cytoplasmic glycoproteins and nucleic acids. They could be isolated and characterized separately.

DISCUSSION

From the results of analysis of the AIR given in Table 1 for immature and mature runner beans and those for different tissues of the tea plant [3, 4], it is clear that the fractionation and isolation of the various groups of polysaccharides from AIR is fraught with many difficulties and complications because of co-precipitation effects. The co-precipitated compounds will be present as impurities in the isolated polysaccharide fractions. The work by Mares and Stone [22] with wheat endosperm and, to a certain extent, that of Knee [5] with apple fruit cell walls also bears this out. The association of the protein with the polysaccharide may result in some non-specific steric inhibition of periodate oxidation as the latter reaction is known to be sensitive to the presence of bulky groups [23]. Also the arabinose and galactose containing cytoplasmic proteins would tend to obscure the significance of the polysaccharides (e.g. pectic substances) containing these sugars. Furthermore, most of the co-precipitated polyphenols are solubilized during the "delignification step" along with the lignin. This would result in the solubilization of an indefinite amount of co-precipitated proteins as well, thereby preventing an approximate estimate of the lignin content of the tissue from the difference in weights before and after the "delignification step". Therefore, unless the cell wall preparation is known to be homogenous, undegraded and free of adsorbed cytoplasmic constituents, caution must be exercised in interpreting any evidence obtained from such an analysis.

Preliminary experiments with the AIR showed that the alcohol precipitated proteins (and polyphenols) cannot readily be solubilized by treatment with powerful protein solubilizing reagents like SDS, SDC, PAW, SDC followed by PAW and pronase. These findings underlined the inherent dangers of dehydrating the tissue with either alcohol or Me_2CO. The effect of alcohol and other factors affecting the co-precipitation of phosphate compounds with the AIR has been discussed elsewhere [11].

It was clear at this stage that the fresh tissue had to be extracted. Any form of drying was avoided to minimize undesirable chemical changes such as production of artifact lignin via the non-enzymic browning reaction [24]. Because boiling SDS has been used for preparing "protein free" fibre from air dried plant tissues [15], SDS and other solvents such as SDC and PAW were tried. The last two solvents have been used for isolation of bulk proteins from leaves [16, 17]. The extractions were carried out in the cold to "arrest" enzymic activity and minimize solubilization of pectic substances. The results given in Tables 2 and 3 show that PAW was more effective than SDS or SDC and that sequential extraction of the fresh ball-milled tissue with SDC and PAW gave wall preparations containing the least amount of proteins. Because the residual proteins are rich in hydroxyproline (which is a characteristic feature of cell wall proteins [19–21]) and cannot be solubilized by treatment with pronase, it seems reasonable to assume that they are cell wall proteins and that the wall preparations are relatively "clean". Therefore the above procedure was adopted for preparation of cell wall material. Two stage extraction of the tissue, i.e. SDC followed by PAW, was preferred instead of direct extraction with PAW for the following reasons: (1) much less (about a fifth) PAW is required

and the SDC extracted tissue could be easily handled; (2) ball-milling and handling tissues extracted with PAW proved very difficult because of the toxic nature of the solvent; (3) the "bulk" proteins and cold water soluble polysaccharides could be easily isolated from SDC extracts; (4) partial fractionation of the proteins is effected. In this method the bulk of the cytoplasmic proteins are removed with SDC and the remaining weakly adsorbed proteins with PAW. The PAW also removes the adsorbed deoxycholate, lipids, residual polyphenols and pigments. This extraction is the result of the strong hydrogen-bond-breaking and dissociating action of phenol [25]. The proteins solubilized by PAW contained appreciable quantities of hydroxyproline (though much less than that of the final residue) and gave mainly arabinose and galactose on hydrolysis. Therefore they appear to be glycoproteins and may be involved in cell wall metabolism. It is possible that they are (weakly) bound cell wall enzymes [26, 27]. Trials with starch, pectin (polygalacturonic acid) and Sigma α-cellulose showed that these polymers are not solubilized by PAW. Ball-milling the macerates for 15–20 hr not only ensured almost complete disruption of the cells and organelles like chloroplasts but also finely ground even heavily lignified tissues like "strings" and "parchment layer" from mature beans. This allowed the solvents to penetrate the sample fully and dissolve the cytoplasmic constituents. Further, preliminary studies showed that unless the tissue was ball-milled, there was incomplete removal of cytoplasmic proteins (especially from mature tissues) and the digestibility of the starch with the α-amylase was much less. Excessive ball-milling (periods >24 hr) should be avoided (especially with parenchyma) as this would result in the production of very finely ground tissue, which cannot readily be separated by centrifugation.

The use of α-amylase to remove starch from the cholate extracted residue or the final residue completes the purification of cell wall material. The quantity of α-amylase required for removal of starch would depend very largely on the starch content of the tissue and must be worked out for each material.

It is useful to note that most, if not all, of the cytoplasmic proteins have been removed from the final cell wall preparation. Therefore the possible contamination with co-precipitated polyphenols would be minimal. Hence the difference in weight of the pectate extracted material before and after the delignification step would give a fair estimate of the lignin content of the tissue, provided a correction is made for the solubilized cell wall protein. Further, there should be a close relation between the total nitrogen of the final residue and the total cell wall protein.

The improved method described in this paper makes it possible to study in a comprehensive manner the changes in the cell wall constituents of vegetables during growth, maturation and senescence. The method has been used to isolate "pure" cell wall preparations from whole beans and different tissues (parenchyma, "strings" and "parchment layer") of mature beans. Fractionation studies on the purified wall preparations revealed some interesting characteristics about the nature of the wall proteins and the polymers with which they are associated and throws more light on the composition of wall polysaccharides. These will be the subject of later papers.

EXPERIMENTAL

Chemicals. The pronase and α-amylase were purchased commercially.

Plant material. Immature (av. length and width 15 and 1 cm respectively) and mature (av. length and width 30 and 2 cm respectively) runner beans (var. Streamline), asparagus shoots (var. Conovors Colossal) and potatoes (var. King Edward) used in this investigation were collected from plants grown in experimental plots near the laboratory.

Fractionation and analysis of alcohol-insoluble residue (AIR). The AIR was first extracted with $CHCl_3$–MeOH (2:1) for 2 hr to remove residual lipids. It was then extracted with sodium hexametaphosphate (2%) to extract the pectic substances according to Stoddart *et al.* [28]. Phosphate extract was dialysed for 3 days to remove most of the metaphosphate and the pectates were ppd with alcohol. An appreciable quantity of the metaphosphate was co-precipitated with the pectic substances and a correction for the co-precipitated phosphate was made when calculating the true weight of the pectic substances. The residue after extraction of the pectic substances was delignified and the hemicelluloses and α-cellulose isolated by methods described before [1]. After each fractionation, the quantitative yield of residue was determined and aliquots taken for dry wt. None of these fractions was corrected for the mineral matter present.

Attempts to remove co-precipitated cytoplasmic proteins from AIR. Preliminary experiments were carried out in an attempt to remove the co-precipitated cytoplasmic proteins from the AIR by treatment with either pronase or protein solubilizing solvents like 1·5% (w/v) SDS, 1% (w/v) SDC or PAW. (a) *Pronase digestion.* The AIR and "purified cell wall material" of beans were finely ground and the powders treated with pronase essentially according to the procedure of Ozeki and Yosizaura

[29]. (b) *Extraction with SDS, SDC and PAW.* The same procedure was used with all 3 solvents. AIR (1 g) was treated with H_2O (10 ml) and allowed to stand at room temp for 18 hr. It was then extracted (stirred for 1 hr) 2× with 50 ml portions of the solvent. The suspension was centrifuged and the residue washed with H_2O, absolute alcohol and Et_2O and dried under reduced pressure over Si gel. The supernatant was analyzed for solubilized proteins and polysaccharides. PAW soluble proteins were isolated according to Jennings *et al.* [17].

Amino acids of protein hydrolysates. Dry samples (4–10 mg depending on the N_2 content) were hydrolyzed at 110° for 24 hr with constant boiling HCl (4 ml) in sealed evacuated tubes. Acid was removed by evapn and amino acid analyses carried out with an automatic analyzer. In the above analyzer, the hydroxyproline and aspartic acid peaks overlapped. However, because the ninhydrin derivative of hydroxyproline has a maximum at 440 nm and that of aspartic acid at 570 nm, it was possible to resolve these overlapping peaks from a knowledge of the 440 and 570 nm contributions [30]. Trials with mixtures of authentic hydroxyproline and aspartic acid gave recoveries greater than 90%. In the preliminary experiments, the amino acids were detected by 2D PC [12]. The occurrence of hydroxyproline in the hydrolysates was detected by spraying with ninhydrin-isatin [31].

Sugars present in hydrolysates of polysaccharides. The polysaccharide fractions were hydrolyzed with 2 N H_2SO_4 and sugars isolated by the methods described before and detected after 1D PC using BuOH–C_5H_5N–H_2O (6:4:3) and aniline phthalate spray [32].

Total nitrogen and "protein". This was determined by the micro-Kjeldahl digestion followed by distn and titration and expressed as "protein" after multiplication by the factor 6·25. In the case of the AIR the "protein figure" would include a small contribution from the precipitated nucleic acids. However, the "purified cell wall material", i.e. the residue obtained after sequential extrn with SDC and PAW, had negligible or no contamination with nucleic acids. In fact the "protein nitrogen" obtained on addition of amino nitrogen from the Beckman column accounted for 85–90% of the nitrogen content of the cell wall material.

Phosphorus. The hexametaphosphate co-precipitated with the pectic substances was determined by the method of Allen [33].

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REFERENCES

1. Jermyn, M. A. and Isherwood, F. A. (1956) *Biochem. J.* **64,** 123.
2. Reid, J. S. G. and Wilkie, K. C. B. (1969) *Phytochemistry* **8,** 2053.
3. Selvendran, R. R. and Selvendran, S. (1972) *Phytochemistry* **11,** 3167.
4. Selvendran, R. R., Perera, B. P. M. and Selvendran, S. (1972) *J. Sci. Food Agr.* **23,** 119.
5. Knee, M. (1973) *Phytochemistry* **12,** 637.
6. Henderson, G. A. and Hay, G. W. (1972) *Carbohyd. Res.* **23,** 379.
7. Thornber, J. P. and Northcote, D. H. (1962) *Biochem. J.* **82,** 340.
8. Newcomb, E. H. (1963) *Ann. Rev. Plant Physiol.* **14,** 43.
9. Loomis, W. D. and Battaile, J. (1966) *Phytochemistry* **5,** 423.
10. Cherry, J. H. and Chroboczek, H. (1966) *Phytochemistry* **5,** 411.
11. Isherwood, F. A. and Barrett, F. C. (1967) *Biochem. J.* **104,** 922.
12. Selvendran, R. R. and Selvendran, S. (1973) *J. Sci. Food Agr.* **24,** 161.
13. Burton, W. G. (1966) in *The Potato*, p. 146, Veenman, H. & Zonen, N. V., Wageningen, Holland.
14. Lamport, D. T. A. and Northcote, D. H. (1960) *Nature*, **188,** 665.
15. Van Soest, P. J. and Wine, R. H. (1967) *J. Assoc. Offic. Agr. Chemists* **50,** 50.
16. Betschart, A. and Kinsella, J. E. (1973) *J. Agr. Food Chem.* **21,** 60.
17. Jennings, A. C., Pusztai, A., Synge, R. L. M. and Watt, W. B. (1968) *J. Sci. Food Agr.* **19,** 203.
18. Street, H. V. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 859, Academic Press, New York.
19. Heath, M. F. and Northcote, D. H. (1971) *Biochem. J.* **125,** 953.
20. Lamport, D. T. A. (1965) in *Advances in Botanical Research* 2, (Preston, R. D., ed.), p. 151, Academic Press, London.
21. Lamport, D. T. A. (1970) *Ann. Rev. Plant Physiol.* **21,** 235.
22. Mares, D. J. and Stone, B. A. (1973) *Aust. J. Biol. Sci.* **26,** 813.
23. Garner, E. F., Goldstein, I. J., Montgomery, R. and Smith, F. (1958) *J. Am. Chem. Soc.* **80,** 1206.
24. Van Soest, P. J. (1965) *J. Assoc. Offic. Agr. Chemists* **48,** 785.
25. Bagasarian, M., Matheson, N. A., Synge, R. L. M. and Youngson, M. A. (1964) *Biochem. J.* **91,** 91.
26. Ridge, I. and Osborne, D. J. (1970) *J. Exp. Botany* **21,** 843.
27. Klis, F. M., Dalheizen, R. and Sol, K. (1974) *Phytochemistry* **13,** 55.
28. Stoddart, R. W., Barrett, A. J. and Northcote, D. H. (1967) *Biochem. J.* **102,** 194.
29. Ozeki, T. and Yosizawa, Z. (1971) *Arch. Biochem. Biophys.* **142,** 177.
30. Boulter, D. in *Techniques in Amino Acid Analysis* (Schmidt, D. J., ed.), p. 155, Technicon International Division SA, Geneva, Switzerland.
31. Kolor, M. G. and Roberts, H. R. (1957) *Arch. Biochem. Biophys.* **142,** 177.
32. Wilson, C. M. (1959) *Analyt. Chem.* **31,** 1199.
33. Allen, R. J. L. (1940) *Biochem. J.* **34,** 858.