Pergamon

0031-9422(95)00675-3

Phytochemistry, Vol. 41, No. 3, pp. 719–723, 1996

0031-9422/96 $15.00 + 0.00

SOLUBILIZATION OF PECTIC POLYSACCHARIDES FROM THE CELL WALLS OF *LEMNA MINOR* AND *APIUM GRAVEOLENS*

PAUL K. KINDEL, LIANG CHENG and BRANDI R. ADE

Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319, U.S.A.

(*Received in revised form* 15 *July* 1995)

Key Word Index—*Lemna minor*; Lemnaceae; duckweed; *Apium graveolens*; Umbelliferae; celery; cell wall; pectic polysaccharides.

Abstract—The kinetics of solubilization of pectic polysaccharides from purified cell walls of *Lemna minor* (duckweed) and *Apium graveolens* (celery) by ammonium oxalate at 22° was determined. With cell walls of *L. minor*, 30 ± 1 (average of three experiments, average deviation from the mean) and 34 ± 1% of the total anhydrouronic acid of the cell walls was solubilized in 15 min and 5 hr, respectively. Water at 22° solubilized 1.3 ± 0.2% and 1.3 ± 0.4% of the total anhydrouronic acid in 15 min and 5 hr, respectively. With cell walls of *A. graveolens*, 19 ± 1% (average of two experiments, average deviation from the mean) and 23 ± 1% of the total anhydrouronic acid of the cell wall was solubilized in 15 min and 3 hr, respectively. Water at 22° solubilized 0.9 ± 0.6 and 0.9 ± 0.1% of the total anhydrouronic acid in 15 min and 3 hr, respectively. When *trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid was the extractant, recovery values based on weight for the soluble pectic polysaccharide fractions were incorrect (high). Color formation in the uronic acid test was inhibited by sodium dodecyl sulfate. A rapid, two-step procedure for isolating purified cell walls was developed.

INTRODUCTION

The primary cell wall of plants maintains the structure of growing plant cells and is involved in a variety of important biological processes [1]. Pectic polysaccharides* are one of the three polysaccharide constituents of primary cell walls. Little is known about the structural role of pectic polysaccharides in cell walls or about their biosynthesis. Understanding these requires that the structure of the pectic polysaccharides be known. The complete structure of any pectic polysaccharide has not been determined and even less is known about the degree of structural variation in the total complement of pectic polysaccharides present in any one plant. In order to obtain accurate structural information a suitable isolation procedure must be used.

The amount of pectic polysaccharides solubilized from cell walls is dependent both on the origin of the cell walls and on the method of extraction [2–15]. A significant portion of the cell wall pectic polysaccharides is readily solubilized by chelating agents and the extent of their solubilization is easily followed colorimetrically. Surprisingly, the time course of solubilization of pectic polysaccharides from purified cell walls at 22° has not been determined. Experiments of this type will show how distinct the difference in solubility is between cell wall pectic polysaccharides readily solubilized and those that tend to remain in the cell wall. They will also permit rational selection of the shortest possible extraction time needed for complete solubilization of a fraction. Our main goal was to determine the kinetics of solubilization of pectic polysaccharides from purified cell walls of *Lemna minor* (duckweed) and *Apium graveolens* (celery).

Pure cell walls are needed before the kinetics of solubilization of pectic polysaccharides can be determined. Both organic [16, 17] and aqueous solvents have been used in the isolation of cell walls, and both have their disadvantages. When aqueous solvents are used cell wall constituents may be solubilized or enzymatically degraded or both. The solubilization of cell wall pectic polysaccharides during the preparation of cell walls has been reported; however, in no case was the solubilized material firmly identified [2–8]. Ionic detergents such as sodium dodecyl sulfate (SDS) have been used in the isolation of cell walls [18], but we found that SDS binds to isolated cell walls and subsequently interferes with color development in the uronic acid assay (this paper). Our second goal was to develop an aqueous cell wall isolation procedure that was rapid, mild, simple and yielded pure cell walls in good yield.

---

*Pectic polysaccharides are defined as those containing mainly galacturonic acid units in the main chain (backbone) of the polysaccharide.

RESULTS

Characterization of cell walls

Cell walls prepared from *L. minor* were dark green after treatment with a Waring blender but pale green to white after treatment with both a Waring blender and a French press. This indicated numerous intact cells with intact chloroplasts were present after the homogenization step but few were present after both steps. Examination of both preparations by both regular and confocal light microscopy confirmed that this was the case. Examination of the final cell wall preparation by electron microscopy showed only cell walls and an occasional thick-walled cell were present. Membranes were not observed adhering to cell walls. The final celery cell wall preparations were cream-colored. Some small multicellular pieces of tissue were observed in the celery preparations and based on examination by light microscopy we estimated 80–85% of the cells were broken. The protein content of purified cell walls of *L. minor* and *A. graveolens* was 5.5% and 1.0%, by weight, respectively. Purified cell walls from other plants have similar protein contents [19]. The uronic acid content of the cell walls of *L. minor* and *A. graveolens* was 189 ± 10 mg of anhydrouronic acid per gram of dry cell walls (mean of three experiments) and 251 ± 10 mg per gram (two experiments), respectively.

Solubilization of cell wall pectic polysaccharides

The average results of three experiments measuring the time course of solubilization of pectic polysaccharides from purified cell walls of *L. minor* at 22° with 0.05 M ammonium oxalate (pH 5.5) are shown in Fig. 1. On average 30 ± 1% (average deviation from the mean) of the total anhydrouronic acid of the cell walls was solubilized in 15 min and 33 ± 1% (two experiments for this value), 30 ± 3%, and 34 ± 1% was solubilized in 1, 3 and 5 hr, respectively. Water at 22° solubilized 1.3 ± 0.2% of the total anhydrouronic acid of the cell walls in 15 min and 1.3 ± 0.4% in 5 hr. The total amount of anhydrouronic acid recovered in the 15 min and 5 hr ammonium oxalate soluble fractions and the corresponding insoluble residue fractions was 99 ± 5% and 101 ± 4%, respectively. The total cell wall material solubilized by ammonium oxalate in 15 min and 5 hr was 7.9 ± 1.6% and 6.2 ± 1.2%, respectively. These values are based on the dry weights of the starting cell walls and recovered ammonium oxalate residues.

The average results from two experiments measuring the time course of solubilization of pectic polysaccharides from purified cell walls of *A. graveolens* by 0.05 M ammonium oxalate (pH 5.5) at 22° showed that 19 ± 1%, 22 ± 1%, 22 ± 1%, and 23 ± 1% of the total anhydrouronic acid of the cell walls was solubilized in 15 min, 1 hr, 2 hr and 3 hr, respectively (Fig. 1). Less than 1% of the total anhydrouronic acid of the cell walls was solubilized by 15 min and 3 hr extractions with water at 22°. In the above experiments, anhydrouronic acid values were normalized on the basis of 1 g dry weight of cell walls extracted and then percentage values were calculated. Percent values are relative to the anhydrouronic acid content of dry cell walls, which was set equal to 100%.

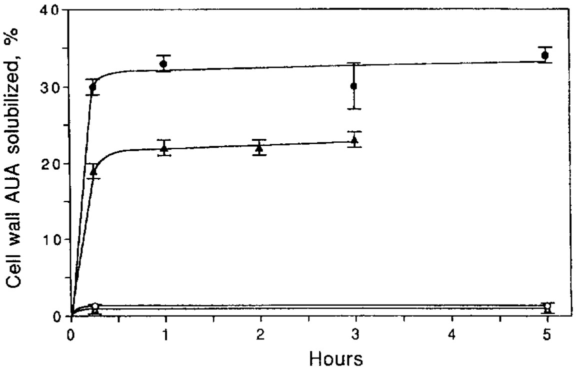

Fig. 1. Time course of solubilization of pectic polysaccharides from purified cell walls *L. minor* (average of three experiments) and *A. graveolens* (two experiments). Purified cell walls of *L. minor* and *A. graveolens* were extracted with ammonium oxalate (●, *L. minor*; ▲, *A. graveolens*) and water (○, *L. minor*; △, *A. graveolens*) as described in the Experimental section. The anhydrouronic acid contents of the dry cell walls and the method of calculating percent values are given in the Results. Dry weights of cell walls were calculated from wet weights and the wet weight/dry weight ratio of the cell walls. For *L. minor* the average wet-weight/dry-weight ratio of the cell walls was 15.3 ± 2.4; for *A. graveolens* it was 12.7 ± 0.4. Error bars show the average deviation from the mean. AUA, anhydrouronic acid.

Analysis of filtrates from the preparation of cell walls for uronic acid

The amount of anhydrouronic acid in the combined, dialysed filtrates obtained during the preparation of cell walls of *L. minor* and *A. graveolens* was 13% (three experiments) and 5.0% (two experiments), respectively, of the total anhydrouronic acid present in the combined filtrates plus cell walls. Percent values were calculated from anhydrouronic acid values normalized on the basis of 1 g dry weight of cell walls extracted.

Effect of various chemicals on the uronic acid test

HCl (1 M), NaCl (1 M) and ammonium oxalate (0.05 M) present individually in samples containing known amounts of galacturonic acid had no effect on color formation in the uronic acid test; control samples were in water. In contrast, 0.0031% and 0.031% (w/v) SDS present in a galacturonic acid solution inhibited color formation 39 and 94%, respectively.

When SDS was used in the isolation of *L. minor* cell walls, measurements of uronic acid in the ammonium oxalate extract were low. Whole plants were homogenized and to one half of the homogenate SDS was added to a concentration of 0.52% (w/v) and to the other half water was added. After stirring for 15 min and centrifuging, the cell wall fraction was washed once with 0.1 M NaCl and once with water and then extracted twice with ammonium oxalate, each time for 4 hr. The amount of uronic acid detected in the ammonium oxalate soluble extracts from cell walls prepared with SDS present, per gram dry weight of cell walls, was 44% (average of two experiments) of that in the water control.

Extraction of cell walls with trans-1,2-*diaminocyclohexane-N,N,N′,N′-tetraacetic acid* (*CDTA*)

Individual samples of cell walls of *L. minor* were extracted with CDTA and ammonium oxalate for 15 min and 5 hr at 22°. The chelator-soluble fractions were dialyzed for 21 hr in water with six changes and dried to constant weight. The dry weight of the 15 min and 5 hr fractions solubilized by CDTA was 4.7 times and 4.8 times greater, respectively, than the corresponding ammonium oxalate fractions. The values were normalized on a per gram dry weight of cell walls extracted. The anhydrouronic acid and total sugar content of the two extracts obtained at each extraction time were basically the same.

DISCUSSION

The results in Fig. 1 show there is a rapid solubilization of pectic polysaccharides from purified cell walls of *L. minor* and *A. graveolens* by ammonium oxalate at 22° and then the rate of solubilization decreases sharply until solubilization stops. The time needed for complete solubilization of the 22° ammonium oxalate-soluble fraction was approximately 30 min; there was, however, only a slight increase in the percent solubilized between 15 min and 1 hr (Fig. 1). The almost complete cessation of solubilization showed that the difference in solubility between the soluble and insoluble pectic polysaccharides is distinct. When impure preparations of cell walls of *L. minor* were used (preparations obtained following the Waring blender step), the rate of solubilization was not as rapid as that shown in Fig. 1 and pectic polysaccharides continued to be released for the entire 5 hr (data not presented). This result shows the importance of using purified cell walls. We conclude that the chelator-soluble pectic polysaccharides are held in the cell wall by ionic interactions alone.

Extraction times of 1.25–6.5 hr have been used to solubilize the 22° chelator-soluble cell wall fraction [2, 8, 9, 12, 14]. Our results show that such long extraction times are unnecessary and are probably undesirable because of the possibility of polysaccharide degradation. The results in Fig. 1 show that a 30 min extraction with ammonium oxalate at 22° is sufficient to solubilize the chelator-soluble fraction, at least with the two cell walls tested. Since a rapidly-solubilized pectic polysaccharide fraction is present in both a monocot and a dicot, this type of fraction may be present in the cell walls of many higher plants.

The extent to which cell wall pectic polysaccharides are solubilized during the preparation of cell walls is not known. Our results with *L. minor* and *A. graveolens* show that 13% and 5.0%, respectively, of the total uronic acid units present in the cell walls plus filtrates were present in the filtrates. It was not established if the uronic acid material in the filtrates originated from the cell walls. However, no significant amounts of pectic polysaccharides were solubilized by water once the cell walls were isolated (Fig. 1).

HCl and NaCl at 1 M and ammonium oxalate at 0.05 M in the sample solution do not interfere, with color formation in the uronic acid test, but SDS does. CDTA, when used as the extractant, was not completely removed from the 22° chelator-soluble fraction by dialysis in water and therefore incorrect (high) dry weights of the fraction were obtained (data not presented). Similar results have been reported by others [20].

The findings that the 22° chelator-soluble pectic polysaccharide fraction of plant cell walls was solubilized in a short time – slightly more than 15 min – and that solubilization of cell wall material virtually ceased after this time period have not been reported previously. The time needed for solubilization of the 22° chelator-soluble fraction was the same for the two plants. In each plant a specific percentage of the total cell wall pectic polysaccharides was solubilized, however, the percent solubilized was different for the two plants. These findings were obtained only when purified cell walls were used.

EXPERIMENTAL

Materials and general methods. Lemna minor was grown as described elsewhere [21]. *Apium graveolens* was purchased from a local grocery. Dialysis tubing with a 6000–8000 M_r cutoff was from Spectrum Medical Industries, Inc. Nylon mesh (3–15/6) was from Tetko, Inc. Uronic acid and total sugar were determined with 3-hydroxydiphenyl [22] and phenol-sulfuric acid [23], respectively. The determinations of uronic acid and neutral sugar in the combined filtrates were corrected for mutual interference. Equations based on Beer's law were derived for calculating the concn of each sugar type measured in the presence of the interfering sugar type. For the calculations, D-galacturonic acid monohydrate and sucrose were used as the representative uronic acid and neutral sugar types, respectively. The extinction coefficients of these two in both sugar tests were determined and were used in the equations. Bovine serum albumin was used as the protein standard. Nitrogen by the Kjeldahl method was determined by Galbraith Laboratories and the protein content was calculated by multiplying by 6.25. Samples were dried to constant weight under vacuum and over P_2O_5 at 22°. Cell wall prepns were examined by transmitted brightfield microscopy with a standard light microscope and those from *L. minor* were further examined by laser scanning transmitted brightfield microscopy with a Zeiss 10 laser scanning microscope. Cell wall prepns of *L. minor* were also examined by transmission electron microscopy at 10000 to 59000 × with a Phillips CM 10 electron microscope operated at 100 kV. Samples were fixed with glutaraldehyde and O_sO_4 and infiltrated with Quetol 651-vinylcyclohexane dioxide [24]. The sample sections were 90 nm thick and were stained with uranium acetate and lead citrate.

Preparation of cell walls and rate of solubilization of pectic polysaccharides. An experiment with *L. minor* is described; however, the same procedure was used with *A. graveolens* except one extraction time was different. Whole plants of *L. minor* were suspended in water at 22° and the water was decanted. Washing was repeated four times. Plants were freed of excess water with absorbent paper and weighted wet (52 g). Petioles of *A. graveolens* were diced at 4° to cubes 0.5–1.0 cm on a side.

The following was performed at 4°. Plants (47.7 g, wet wt) were suspended in 120 ml of 1.0 M NaCl and homogenized for four, 40-s periods in a Waring blender with 10 sec intervals between periods. The suspension was filtered with 15 μm Nylon mesh, the particulate material was washed with 75 ml of water and the combined filtrates were saved. The cell walls were resuspended in 120 ml of water and passed through a French pressure cell at an average cell pressure of 16 000 pounds/in^2. The suspension was filtered through 15 μm Nylon mesh, the cell walls were washed with 75 ml of water and the combined filtrates were saved. The cell walls were weighed (9.3805 g, wet wt) and examined microscopically.

Two weighed portions of cell walls, each 0.4 ± 0.04 g, wet wt, were dried to constant weight and two weighed portions, each 0.3 ± 0.04 g, wet wt, were hydrolysed as described below. The remainder of the cell walls was divided approximately equally between six, 50 ml beakers (each sample was about 0.85 ± 0.05 g, wet wt) and the wet weights recorded. Four of the cell wall portions were suspended in 0.05 M ammonium oxalate (pH 5.5) (8.5 ml per sample) and stirred for 15 min, 1, 3, and 5 hr at 22°. Two portions were suspended in water (8.5 ml per sample) and stirred for 15 min or 5 hr at 22°. The suspensions were centrifuged at 4° and the supernatant solutions were filtered. The residues were suspended in water and the suspensions were centrifuged and the supernatant solutions filtered. The washing was repeated once and the three filtrates were combined and analysed for uronic acid.

One weighed portion of the washed residue from both the 15 min and 5 hr ammonium oxalate extractions, each approximately 0.3 g, wet wt, was hydrolysed and a second weighed portion (about 0.4 g, wet wt) of each was dried to constant weight. Hydrolysis of samples was by refluxing with 20 ml of 0.25 N HCl for 3 hr at 100° [25]. The hydrolysed samples at 22° were brought to pH 7 with 2.0 N KOH/0.5 M ammonium oxalate and stirred for 10 min. The samples were centrifuged at 4° and the supernatant solutions were filtered. The precipitates were resuspended in water, the suspensions filtered, and the appropriate filtrates combined and analyzed for uronic acid. The results were normalized on a per gram dry wt of cell walls basis and then converted to percent (Fig. 1).

Analysis of the combined filtrates from the preparation of cell walls for sugars. The volumes of the two combined filtrates obtained in the above procedure for the prepn of cell walls were measured. A portion of each was centrifuged and the supernatant solutions were filtered through separate 5.0 μm MF-Millipore filters. The two samples were dialysed in water for 26 hr, their volumes were measured and they were assayed for uronic acid and total sugar.

Acknowledgements—We thank Drs Joanne Whallon and John W. Heckman of the MSU Laser Scanning Microscope Laboratory and the Center for Electron Optics, respectively, for microscope work.

REFERENCES

1. Carpita, N. C., and Gibeaut, D. M. (1993) *Plant* **3**, 1.
2. Jarvis, M. C. (1982) *Planta* **154**, 344.
3. Stevens, B. J. H. and Selvendran, R. R. (1980) *J. Sci. Food Agric.* **31**, 1257.
4. Ring, S. G. and Selvendran, R. R. (1981) *Phytochem.* **20**, 2511.
5. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.* **128**, 321.
6. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.* **135**, 155.
7. Redgwell, R. J. and Selvendran, R. R. (1986) *Carbohydr. Res.* **157**, 183.
8. Ryden, P. and Selvendran, R. R. (1990) *Biochem. J.* **269**, 393.
9. Souty, M., Thibault, J.-F., Navarro-Garcia, G., Lopez-Roca, J.-M. and Breuils, L. (1981) *Sci. des Alim.* **1**, 67.
10. De Vries, J. A., Voragen, A. G. J., Rambouts, F. M. and Pilnik, W. (1981) *Carbohydr. Polymers* **1**, 117.
11. Aspinall, G. O. and Fanous, H. K. (1984) *Carbohydr. Polymers* **4**, 193.
12. Sauliner, L. and Thibault, J.-F. (1987) *Carbohydr. Polymers* **7**, 329.
13. Marcelin, O., Sauliner, L. and Brillouet, J.-M. (1991) *Carbohydr. Res.* **212**, 159.
14. Ryden, P. and Selvendran, R. R. (1990) *Carbohydr. Res.* **195**, 257.
15. Jarvis, M. C., Hall, M. A., Threlfall, D. R. and Friend, J. (1981) *Planta* **152**, 93.
16. Selvendran, R. R. (1975) *Phytochem.* **14**, 1011.
17. Selvendran, R. R. and DuPont, M. S. (1980) *J. Sci. Food Agric.* **31**, 1173.
18. Selvendran, R. R. and O'Neill, M. A. (1987) in *Methods of Biochemical Analysis* (Glick, D., ed.), Vol. 32, p. 25, John Wiley and Sons, New York.
19. Harris, P. J. (1983) in *Isolation of Membranes and Organelles from Plant Cells* (Hall, J. L. and Moore, A. L., eds.), p. 25, *Academic Press*, New York.
20. Mort, A. J., Moerschbacher, B. M., Pierce, M. L. and Maness, N. O. (1991) *Carbohydr. Res.* **215**, 217.
21. Kindel, P. K. and Watson, R. R. (1973) *Biochem. J.* **133**, 227.

22. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484.
23. DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350.
24. Kushida, H. (1980) *J. Electron Microsc.* **29**, 193.
25. Jarvis, M. C., Forsyth, W. and Duncan, H. J. (1988) *Plant Physiol.* **88**, 309.