Isolation and Characterization of Arabinogalactan Proteins Released by Cellulase Digestion of Cabbage Leaves

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A new type of arabinogalactan protein (AGP) tightly associated with cellulose microfibrils and pectic substances was found to be released by cellulase digestion of the insoluble residues given from cabbage leaves after extractions with water and hot water. The content of AGPs in head leaves of cabbage was 2.5-fold higher than that of green leaves. These AGPs were fractionated into five components (C-II$_1$−C-II$_5$) by combination of gel filtration on Sepharose CL-4B and ion-exchange chromatography on DEAE-Toyopearl. The C-II$_1$ fraction eluted from a DEAE-Toyopearl column at 0.07 M NaCl was a typical AGP which is rich in hydroxyproline (28.3%) and consistis of protein (15.4%), neutral sugar (78.8%) with an arabinose/galactose ratio of 1.6:1, and uronic acid (5.8%). The chemical composition analysis of the other four glycoproteins (C-II$_2$−C-II$_5$) eluted at 0.15, 0.175, 0.2, and 0.3 M NaCl, respectively, indicates that these AGPs were complexed with rhamnogalacturonan having wide spectra in protein and pectin contents. The present results furnish AGPs with the structural functions of the primary cell wall in cabbage leaves.

Keywords: *Arabinogalactan protein; hydroxyproline-rich protein; glycoprotein; cabbage; cellulase digestion*

INTRODUCTION

Hydroxyproline-rich glycoproteins (HRGPs) existing in plant cell wall are classified largely into two types with respect to water extractability, and solubility. Arabinogalactan proteins (AGPs) are the major components of the water-soluble HRGP and are widely distributed in the plant kingdom, occurring in leaves, stems, roots, floral parts, and seeds (Clarke et al., 1975, 1978, 1979a,b; Fincher et al., 1983; Showalter and Varner, 1989). AGPs are localized in the extracellular space (apoplast) (Clarke et al., 1975, 1978; Jermyn and Yeow, 1975) and the plasma membrane (Larkin, 1978; Samson et al., 1983; Nothnagel and Lyon, 1986; Norman et al., 1990; Komalavilas et al., 1991; Pennell et al., 1989) with lesser distribution in the cytoplasmic organelle (Mascara and Fincher, 1982; Van Holst et al., 1981). They are also secreted in the medium of suspension-cultured plant cells (Komalavilas et al., 1991; Aspinall et al., 1969; Hori and Sato, 1977; Anderson et al., 1977; Hori et al., 1980; Akiyama and Kato, 1981; Cartier et al., 1987; Gallagher, 1989). The multisite localization of AGPs in plant tissues appears to be analogous to that of some animal proteoglycans, such as heparan sulfate and chondroitin sulfate (Gallagher, 1989).

Many functions have been proposed for AGPs in several biological processes involving plant development, such as recognition of cells and small ligands, cell−cell adhesion, pollen−stigma recognition, water retention, and disease resistance, but none of these functions have been established (Clarke et al., 1979a; Fincher et al., 1983; Showalter and Varner, 1989; Lamport and Northcote, 1960).

On the other hand, extensin is a typical water-insoluble HRGP which is insolubilized by covalent cross-linking to each other through tyrosine residues and ionically interacting with the pectic materials. Previously, it was isolated as a glycopeptide from tomato cultures by trypsin treatment (Lamport, 1969). Later works, however, enabled water-soluble extensin monomers isolated from suspension-cultured media and from cultured cell walls after protease treatment and salt extractions (Ashford and Neuberger, 1980; McNeil et al., 1984). Although both extensin and AGP contain hydroxyproline as the major compositional unit, several compositional differences were noted: (a) extensins were highly basic due to predominating basic amino acids such as lysine and histidine in contrast to AGPs which were acidic and rich in alanine, serine, and threonine, and (b) more than 80% of the carbohydrate portion of extensin was composed of arabinose, while arabinose and galactose were the major sugars in AGPs (McNeil et al., 1984; Cooper et al., 1987). Structural and regulatory functions have been proposed for extensin; a direct precise role in cell wall architecture, however, remains controversial (Esquerre-Tugaye and Mazau, 1981; Fry, 1982; Lamport and Epstein, 1983).

In our previous studies on water-soluble glycoconjugates of vegetables, we developed a new isolation method for AGPs from cabbage and other vegetables and characterized their chemical properties (Yasufuku et al., 1985, 1988). Likewise, we investigated the properties of hot-water-soluble AGP from cabbage (Kido et al., 1991). We proposed that these soluble AGPs be transformed into insoluble forms with leaf hardening (Yasufuku et al., 1987, 1994). A preliminary experiment for release of the insolubilized AGP by protease treatment was found to be ineffective. This may indicate that the insolubilized AGP is firmly embedded with cellulose microfibril networks in cell walls.

In this paper, a method was developed to release a new type of insolubilized AGPs from cabbage leaves by

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treatment with cellulase, and their gross chemical properties were also characterized in relation to their functions in cabbage cell walls.

MATERIALS AND METHODS

Materials. As stated in the previous papers, about 1.5 kg of heads of Rakuyo cabbage (*Brassica oleracea* L. var. Capitata L.) was collected immediately after harvest and separated into head leaves and green leaves free from cores, stalks, and veins (Yasufuku et al., 1985, 1987, 1994). The dry weight of samples was calculated from the moisture contents measured ranging from 92 to 93% by a Cho PD1-240 WH moisture meter at 100 °C. Water-soluble materials containing water-soluble AGPs were removed as described previously (Yasufuku et al., 1985). Cabbage leaves was homogenized by an electric mixer in a 4:3 (v/v) mixture of methanol−water (water content of cabbage leaves was taken as water) and extracted with 8 volumes of chloroform. The residue was once again extracted with the same mixed solvent and further extracted with hot water (80 °C) for 3 h to remove materials containing hot-water-soluble AGPs and pectic substances (Kido et al., 1991). The residues were finally washed with acetone and dried to obtain the extracted residues.

Cellulase from *Aspergillus niger* 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4, practical grade, type I, 0.6 unit/μmol of glucose) was purchased from Sigma Chemical Co., St. Louis, MO. Standard sugars and pullulans (Shodex standard P-52) for gel filtration were obtained at Showa Denko, Co., Ltd., Tokyo, Japan. Sepharose CL-4B, and standard proteins, and HMW kits (67−669 kDa) for electrophoresis were purchased from Phamacia Fine Chemicals AB, Uppsala, Sweden. DEAE-Toyopearl 650 S was purchased from Tosoh Co., Tokyo, Japan. All other chemicals were of analytical reagent grade.

Cellulase Digestion of Insoluble Residues and Purifications of Released AGPs. Dried extracted residue was suspended in 0.5 M sodium acetate buffer, pH 5.0, and digested with cellulase (Sigma) at the enzyme to substrate ratio of 1:750 (dry weight basis). The digestion was carried out at 37 °C for 18 h. The pH of the incubation medium was kept at 5.0 by adding 1.0 N NaOH due to the solubilization of abundant degradation products containing uronic acid. The material released in the medium was recovered by centrifugation, dialyzed against 0.1 M sodium borate buffer, pH 9.0, and gel filtrated on a column (3.5 × 60 cm) of Sepharose CL-4B preequilibrated with the same buffer. Elution was monitored by measuring absorbances at 280 nm for protein and at 480 nm for carbohydrate after development of color by the phenol−sulfuric acid method (Dubois et al., 1951). The elution profile is shown in Figure 1. The C-II fraction containing hydroxyproline as indicated in the figure was further fractionated by ion exchange chromatography on DEAE-Toyopearl 650 S column (1 × 15 cm). As shown in our previous paper for purification of hot-water-soluble AGPs, this column was equilibrated with 0.01 M sodium borate buffer, pH 9.0 (Kido et al., 1991). We found that the fractions eluted with 0.07, 0.15, 0.175, 0.2, and 0.3 M NaCl contained glycoproteins such as AGP or extensin due to the presence of hydroxyproline. These fractions were, therefore, isolated and characterized.

Chemical Analysis and General Methods. Total protein was measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard. Total neutral sugar was measured according to the phenol−sulfuric acid method using a mixture of arabinose and galactose (1:1, w/w) as standard, and the color developed was determined by measuring absorbance at 480 nm (Dubois et al., 1951). Uronic acid was estimated according to the modified carbazole method (Bitter and Muir, 1962). The neutral sugar composition was analyzed according to the postcolumn derivatized method through the use of a Shimadzu LC-5A HPLC on a Shimadzu LC column, ISA-07/S2504, and by RF-530 fluorescence spectrometer at Ex 430 nm and Em 320 nm, after hydrolysis of the sample with 1.0 N sulfuric acid at 100 °C for 6 h. Amino acid composition was analyzed on a Hitachi 835 amino acid analyzer after hydrolysis of the sample with 6.0 N hydrochloric acid at 110 °C for 24 h.

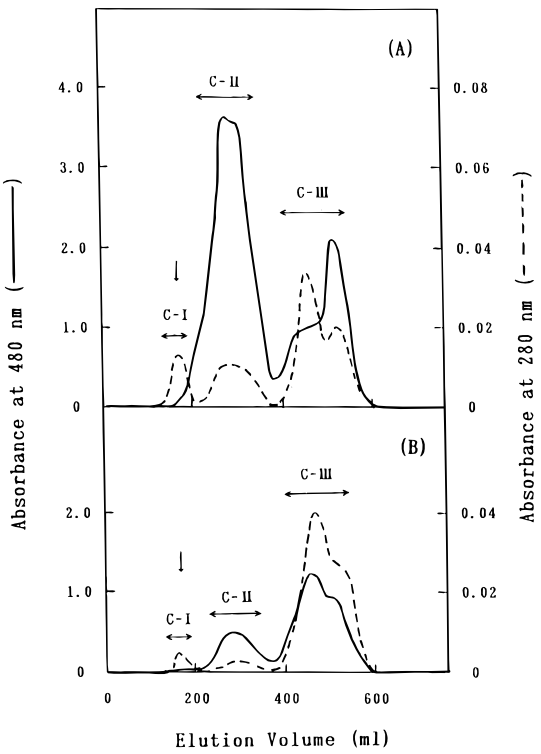

Figure 1. Gel filtration of the materials released by cellulase degradation of head leaves (A) and green leaves (B) of cabbage on a Sepharose CL-4B column. Samples released by cellulase digestion of insoluble residues prepared from 20 g of dried head leaves and green leaves were applied separately on a column (3.5 × 60 cm) equilibrated with 0.1 M sodium borate buffer, pH 9.0. Elution was monitered for carbohydrate (—) and protein (---) as shown in the text. The downward arrow indicates the position of the void volume. The separated fractions obtained from head leaves and green leaves were designated C-I, C-II, and C-III.

Molecular mass of the sample was estimated by gel filtration on a Sepharose CL-4B column (3.5 × 60 cm) using pullulans with known molecular mass (Shodex standard P-82, Showa Denko).

Discontinuous sodium dodecyl sulfate−acrylamide slab gel electrophoresis (SDS−PAGE) was performed using a resolving gel of 5−10% gradient acrylamide with 3.75% acrylamide stacking gel in Tris−glycine buffer, pH 8.6, containing 0.1% SDS (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R-250 for proteins and periodic acid−Schiff reagent for carbohydrates. Standard proteins used for molecular mass estimation were of electrophoresis calibration kit for high molecular weights (Pharmacia AB), consisting of bovine serum albumin (67 000), lactate dehydrogenase (140 000), catalase (232 000), ferritin (440 000), and thyroglobulin (669 000).

RESULTS AND DISCUSSION

Cellulase Digestion of Insoluble Residues from Cabbage Leaves. We found that the crude cellulase preparation from *A. niger* released a substantial amount of hydroxyproline-rich materials from the water-insoluble residue of cabbage leaves. The amount of high molecular mass components released from head leaves (2.9% on the dry weight basis of head leaves) was found to be about 2.5-fold higher than that from green leaves. This value was much higher than those obtained in the water extraction (0.38%) (Yasufuku et al., 1985) and in the hot water extraction (1.5%) (Kido et al., 1991). Hydroxyproline contents in the released materials determined by amino acid analyzer were 18% (head

leaves) and 3% (green leaves) of the total amounts of that originally present in the native leaves. The difficulty of solubilizing HRGPs from the insoluble residues of green leaves by cellulase treatment indicates the tightness of binding of HRGP with cell wall polymers as well as the cross-linkages that occur in the case of extensins. Formerly, seven kinds of proteases and eight kinds of carbohydrate hydrolases were used for the isolation of HRGP from suspension-cultured cells, and extensins were specifically released by proteases (Lamport, 1969; Ashford and Neuberger, 1980). In our preliminary study, trypsin was used for isolation of hydroxyproline-rich materials from the insoluble residues of cabbage leaves. The present cellulase treatment, however, promoted release of more than three times the amount of such materials, indicating the superiority of the present method.

Gel Filtration of the Polymers Released by Cellulase Digestion from Head Leaves and Green Leaves. The released materials from head and green leaves by the cellulase digestion were subjected to gel filtration on Sepharose CL-4B after removal of small molecular weight components by dialysis. Three separated fractions, C-I (130−180 mL), C-II (230−330 mL), and C-III (480−540 mL) were collected, respectively, as shown in Figure 1. Amino acid composition analysis of all fractions indicated that only fraction C-II contained HRGP. On the basis of the high absorbance at 480 nm in C-II, which coincided with the absorbance at 280 nm, it is clear that the amount of HRGP in head leaves is approximately 8-fold higher than that of green leaves.

Polyacrylamide gel electrophoresis indicated that C-II did not penetrate into the separation gel and remained in the stacking gel (results not shown), whereas the molecular mass of C-II estimated by gel filtration was around 180 kDa, similar to that of the water-soluble AGP A-II which was previously isolated from head leaves of cabbage (Yasufuku et al., 1985, 1987). There was no indication of molecular mass difference in the C-II fractions isolated from head and green leaves. Previously, we reported that the water- and hot-water-soluble AGPs (A-I, A-II, and H-II$_3$) with molecular masses ranging from 100 to 400 kDa could penetrate into resolving gel. The AGP (A-I) penetrated slightly into the gel, and A-II and H-II$_3$ penetrated appreciably into the gel with a somewhat broad zone (Yasufuku et al., 1985; Kido et al., 1991). The retention in the stacking gel and high molecular mass (180 kDa) may indicate that the C-II fraction is rich in carbohydrate and present an extremely wide expanded three-dimensional structure in an aqueous solution.

Fractionation of HRGP Released by Cellulase Digestion from Head Leaves and Green Leaves. Further fractionation of C-II was performed using the ion exchange chromatography on DEAE-Toyopearl. The elution was carried out stepwisely using 0.01 M sodium borate buffer, pH 9.0, containing 0.07, 0.15. 0.175, 0.2, and 0.3 M NaCl as shown in Figure 2 to give five fractions (C-II$_1$−C-II$_5$). Table 1 summarizes the yields of the separated glycoproteins on the basis of the dry weight of cabbage leaves. Overlapping of absorbances due to carbohydrate and protein in all C-II subfractions supported their glycoprotein nature.

Chemical Properties of the Isolated HRGPs from Head Leaves. Since the content of the C-II fraction in the green leaves was low (0.05%) and its elution profile on the DEAE-Toyopearl column was similar to that given in the head leaves, chemical

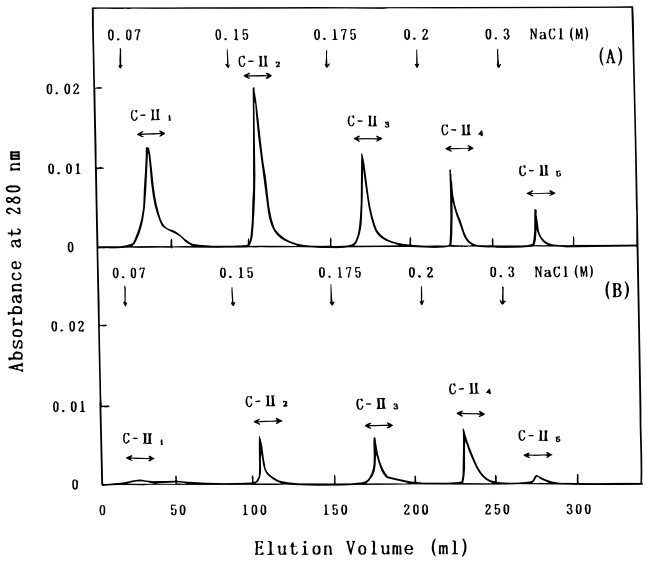

Figure 2. Fractionation of hydroxyproline-containing glycoproteins present in the C-II fractions obtained from head leaves (A) and green leaves (B) of cabbage by DEAE-Toyopearl column. Downward arrows indicate the changing points of elution conditions from 0.01 M sodium borate buffer, pH 9.0, to the same buffer solutions containing 0.07, 0.15, 0.175, 0.2, and 0.3 M NaCl. Elution from a column (1 × 15 cm) was monitered by measuring absorbance at 280 nm. The other experimental conditions were specified in the text. Five separated fractions are designated C-II$_1$, C-II$_2$, C-II$_3$, C-II$_4$, and C-II$_5$.

Table 1. Fractionation of HRGPs in the C-II Fraction Obtained by Cellulase Digestion of Head Leaves and Green Leaves of Cabbage[a]

samples[b]	C-II$_1$	C-II$_2$	C-II$_3$	C-II$_4$	C-II$_5$
head leaves	0.009	0.056	0.025	0.034	0.001
green leaves	tr[c]	0.005	0.019	0.027	tr

[a] Values are expressed as a percentage of the dry weight of cabbage leaves. [b] C-II$_1$, C-II$_2$, C-II$_3$, C-II$_4$, and C-II$_5$ were isolated by elution with 0.07, 0.15, 0.175, 0.2, and 0.3 M NaCl from DEAE-Toyopearl column (Figure 2), respectively. [c] Trace.

Table 2. Chemical Properties of Five AGPs Released by Cellulase Digestion of Head Leaves of Cabbage

components (%)	C-II$_1$	C-II$_2$	C-II$_3$	C-II$_4$	C-II$_5$
composition[a]					
protein	15.4	3.6	1.4	0.6	3.2
neutral sugar	78.8	86.9	84.1	68.5	74.0
uronic acid	5.8	9.5	14.5	30.9	22.8
neutral sugar[b]					
arabinose	59.2	50.9	48.7	36.9	32.6
rhamnose	−[c]	2.2	13.7	28.6	18.0
xylose	3.9	4.8	8.1	14.8	10.0
galactose	36.9	42.1	29.5	19.7	33.4
glucose	−	−	−	−	6.0

[a] Values are expressed as a percentage based on the dry weight of each sample. [b] Relative weight percentages of total sugar. [c] Not determined.

analysis of the separated fractions was carried out with the fractions C-II$_1$−C-II$_5$ isolated from head leaves. The results are listed in Table 2. Their carbohydrate contents were high (85−99%), and their protein contents (0.6−15.4%) and uronic acid contents (5.8−30.9%) were appreciably different. The C-II$_1$ fraction was rich in protein but had the lowest amount of uronic acid, while the fractions C-II$_3$−C-II$_5$ adsorbed strongly on the ion exchange gels were poor in protein but rich in uronic acid. Neutral sugar composition analysis indicated that arabinogalactan was of the common major framework sugars of five separated C-II subfractions. The C-II$_1$

Table 3. Amino Acid Composition of Five AGPs Released by Cellulase Digestion of Head Leaves of Cabbage

amino acid (%)	C-II$_1$	C-II$_2$	C-II$_3$	C-II$_4$	C-II$_5$
hydroxyproline	28.3	12.0	5.1	1.5	2.0
aspartic acid	3.8	6.2	6.9	7.5	7.5
threonine	4.7	5.7	5.2	5.1	5.4
serine	12.3	17.6	17.5	16.3	14.1
glutamic acid	4.8	7.1	8.7	12.3	14.7
proline	5.0	3.1	3.9	2.9	3.5
glycine	8.5	14.5	17.6	20.3	17.6
alanine	4.4	6.7	7.2	7.5	8.4
cystine	0.3	0.7	1.4	0.6	0.3
valine	5.6	5.1	5.2	4.6	5.0
methionine	2.1	0.5	1.0	0.4	0.4
isoleucine	1.5	1.9	3.1	2.7	3.0
leucine	2.3	3.1	4.2	5.2	5.7
tyrosine	5.1	3.4	2.0	2.5	1.9
phenylalanine	1.5	2.1	1.8	2.4	2.0
lysine	5.5	5.1	4.2	3.8	4.0
histidine	3.4	3.2	2.8	2.3	2.0
arginine	1.0	1.8	2.2	2.1	2.5

fraction was free from rhamnose, and the C-II$_2$ fraction contained only a small amount of this sugar. On the other hand, the remaining fractions (C-II$_3$−C-II$_5$) strongly adsorbed on the column were rich in this sugar in accordance with the content of uronic acid. This indicates the coexistence of a rhamnogalacturonan type pectic substance.

Table 3 lists the amino acid composition analysis data. The results indicate that all separated C-II subfractions contained hydroxyproline, with the highest amount in the unadsorbed C-II$_1$ fraction (28.3%) and with lesser amounts (1.5−2.0%) in the strongly adsorbed C-II$_4$ and C-II$_5$ fractions. Serine content was also high in all separated fractions. The contents of glutamic acid and alanine increased with the increase in adsorbability on DEAE-Toyopearl gel. Glycine content was also high in the adsorbed fractions (C-II$_2$−C-II$_5$).

On the basis of the chemical composition analyses, the C-II$_1$ fraction was deduced to be pure AGP. The other four C-II subfractions, C-II$_2$−C-II$_5$, were suggested to be complexes of AGP and rhamnogalacturonan with a wide spectrum of protein and pectin contents.

The requirement of cellulase treatment to release these AGPs and the deficiency of glucose in almost all of these AGPs indicate that they are physically buried firmly inside the cellulose microfibril networks. It was further suggested that AGPs were insolubilized by complex formation with rhamnogalacturonan.

Since rhamnogalacturonan is localized in the primary cell wall, AGP was first concluded to be present in the primary cell wall in association with pectic materials. Water-soluble extracellular and apoplastic AGPs were thoroughly investigated in previous studies. The present results open a new field of research work on AGP in relation to structural and functional aspects of plant cell walls.

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