A Novel Method for Detection of Endo-Xyloglucan Transferase

Kazuhiko Nishitani

Department of Biology, College of Liberal Arts and Sciences, Kagoshima University, Kagoshima, 890 Japan

A new approach has been developed for quantification of the activity of endo-xyloglucan transferase, a novel enzyme that mediates the transfer of a segment of one xyloglucan molecule to another xyloglucan molecule. Purified xyloglucans with defined molecular-weight distributions and their fluorescent derivatives (pyridylamino xyloglucans) were used as substrates for the enzymatic reaction. The transferase activity was quantified by monitoring changes in molecular-weight distributions of substrates by an alkali compatible gel permeation chromatographic system, equipped with a pulsed amperometric detector and a fluorescence detector. This new method was applied to the rapid detection and characterization of a novel transferase derived from plant tissues.

Key words: Cell wall — Endo-xyloglucan transferase — Pulsed amperometry detector — Pyridylamino xyloglucan — Vigna angularis Ohwi et Ohashi (azuki bean) — Xyloglucan.

The plant cell wall is composed of crystalline microfibrils embedded in a matrix of amorphous polymers (Albersheim 1976, Fry 1988). Xyloglucans (XGs) are the major components of the matrix and function as cross-links between cellulose microfibrils, with resultant formation of an interwoven network structure, which provides the wall with viscoelastic properties (Talbott and Ray 1992a). Certain changes in the viscoelastic properties, which are achieved by chemical creepage (Cosgrove 1989), are required for the extension of cell walls and, hence, for cell growth (Masuda 1978, Cleland 1987). Several lines of evidence indicate that structural changes in XGs are involved in such chemical creepage in higher plants (Nishitani and Masuda 1981, 1982, 1983, Inouhe et al. 1984, Hoson et al. 1991). Although involvement of endo-glycosyltransferases in chemical creepage of the wall matrix was postulated almost two decades ago (Albersheim 1976), the existence of such enzymes has not yet been demonstrated. Recently, the existence of some enzyme responsible for transglycosylation between xyloglucan molecules was suggested by circumstantial evidence obtained independently by several research groups (Baydoun and Fry 1989, McDougall and Fry 1990, Nishitani and Tominaga 1991, Fry et al. 1992, Talbott and Ray 1992b). Furthermore, Nishitani and Tominaga (1991) and Talbott and Ray (1992) suggested the involvement of a transferase in regulation of the molecular-weight distribution of xyloglucans in plant cell walls.

In apoplastic solutions isolated from the cell wall space of epicotyls of Vigna angularis, we detected an enzymatic activity that mediated an increase in the molecular weights of XGs in vitro (Nishitani and Tominaga 1991). The enzymatic reaction suggested the presence of an endo-xyloglucan transferase (EXT) in the cell-wall space of the plant. A similar activity was also detected in tissue homogenates from other plants (Fry et al. 1992). The purification and characterization of this activity was, however, unsuccessful, in part because no suitable assay system specific for the transferase was available (Nishitani and Tominaga 1991, Fry et al. 1992). In order to characterize the EXT, we have developed a new assay system, using a fluorescent derivative of XG as the acceptor substrate and an alkali-compatible GPC system. Use of these new tools has made it possible to detect and quantify the activity of a transferase that catalyzes transfer of a large segment of XG from one XG molecule to another molecule to generate a chimeric or recombinant XG molecule. This new procedure was successfully exploited for the first purification of an EXT from plant tissues (Nishitani and Tominaga 1992).

Materials and Methods

Preparation of enzyme—A crude preparation of the
apoplastic enzyme with EXT activity was obtained from epicotyls of dark-grown seedlings of *Vigna angularis* Ohwi et Ohashi cv. Takara by the previously reported method (Nishitani and Tominaga 1991). EXT was purified from the crude preparation as described elsewhere (Nishitani and Tominaga 1992).

**Preparation of substrates**—XGs extracted from cell walls of *Vigna* (Nishitani and Tominaga 1991) and tamarind (*Tamarindus indica* L.) (Fanutti et al. 1991) were further fractionated by GPC on a column (16 mm i.d. × 500 mm) of Superose 6 prep (Pharmacia), with water as the eluent, for preparation of xylolgucans with defined molecular-weight distributions. The reducing ends of purified XGs from tamarind were coupled with 2-aminopyridine by reductive amination using sodium cyanoborohydride (Hase et al. 1979) to generate PA-XGs. The labeled XGs were fractionated and purified by GPC on Superose 6 prep with water as the eluent.

**Enzymic reaction**—Ten µg of non-labeled XGs from *Vigna* or tamarind were incubated in 10 µl of 0.2 M sodium acetate buffer (pH 5.8) that contained various amounts of the crude preparation of enzyme or the purified EXT, at 25°C, for various periods of time. Alternatively, a mixture of 10 µg of non-labeled XG and 2 µg of PA-XG were used as substrate and were incubated with purified EXT under the same conditions.

**HPLC**—Changes in molecular weights of XGs during the enzymatic reaction were analyzed by GPC. The GPC system consisted of an alkali-stable non-metal pump (model GPM; Dionex, U.S.A.), a pulsed amperometric detector (PAD; Dionex), a fluorescence detector (model RF 535; Shimadzu, Japan) and columns of TSK gel G3000PW (8 mm i.d. × 300 mm, in a glass tube) and G5000PW (8 mm i.d. × 300 mm, in a glass tube) connected in series. The glass columns of TSK gels were specially manufactured by Toso (Tokyo, Japan). The operating parameters for the PAD were as follows: E1, 0.05 V for 480 ms; E2, 0.6 V for 120 ms; E3, −0.6 V for 60 ms; pulse duration range switch set at position 2; response time, 1 s. For detection of PA-XGs by the fluorescence detector, an excitation wavelength of 320 nm and an emission wavelength of 400 nm were used. Samples were injected after having been dissolved in 20 µl of a 0.1 M solution of sodium hydroxide. The columns were eluted with a 30 mM solution of sodium hydroxide that contained 15 mM sodium acetate, at a flow rate of 1 ml min⁻¹.

**Molecular-weight calibration**—Pullulans (mol wts, 853,000, 380,000, 186,000, 100,000, 48,000, 23,700, 12,300, 5,800 and 1,810 Da) were manufactured by Hayashibara Biochemical Laboratories (Japan). Maltooligomers (mol wts, 667 and 342 Da) and glucose were individually chromatographed on columns of TSK gels 5000PW and 3000PW connected in series. These carbohydrates were used as markers for GPC. By measuring peak areas of individual carbohydrates, as detected by the PAD, we were able to evaluate responsiveness of the PAD to different carbohydrates relative to its response to glucose.

**Results and Discussion**

**Gel-permeation chromatography**—Figure 1 shows the relationship between log₁₀ (mol wt) and elution volume of authentic carbohydrates passed through columns of TSK gels 5000PW and 3000PW connected in series. A mostly linear relationship between the two parameters was observed over a range of molecular weights from 853,000 to 180 Da.

Figure 2 shows the relative responsiveness of the PAD to individual carbohydrates with different molecular weights. The PAD response per unit weight of carbohydrate, which was defined as PAD responsiveness, declined rapidly as the mol wt increased from 180 to 5,800. However, the PAD responsiveness to large polymers (mol wts, 853,000 to 12,300 Da) was almost constant. Thus, the PAD
Detection of endo-xyloglucan transferase

response can be used as a measure of the total polysaccharide content of the eluate.

The response of the fluorescence detector was proportional to the number of PA-XG molecules because the reducing end of each XG was tagged with a single pyridylamino group. Less than 10 μg of XG or 20 pmole of PA-XG were required for analysis of the distribution of molecular weight by GPC under our conditions.

Peak broadening—Ten μg of non-labeled XG were incubated with 1 μg of the crude preparation of apoplastic enzyme. Analysis of the reaction products by GPC revealed that the enzymatic reaction caused broadening of the peak of XGs without any change in the elution volume associated with the top of the peak. The broadening of the peak indicates that the enzymatic action generated both higher- and lower-molecular-weight species of XG molecules by transglycosylation between XG molecules (Fig. 3).

The broadening of the peak caused by the crude preparation of enzyme was accompanied by generation of monosaccharides (the arrow a in Fig. 3). By contrast, no monosaccharides or oligomers were produced when XG was incubated with the purified EXT, which also mediated broadening of the peak (Fig. 4). Clearly, the monosaccharides were liberated by the action of hydrolases present in the crude preparation of the enzyme and not by the action of EXT. Thus, the peak broadening reflects the transglycosylation reaction that is catalyzed by EXT. Extended incubation for several hours with the crude enzyme caused degradation of high-molecular-weight XGs and interfered

![Fig. 2 Relationship between log_{10} (mol wt) and responsiveness of the PAD to carbohydrates.](image)

Carbohydrates with various molecular weights were chromatographed as described in the legend to Fig. 1 and the peak area for individual carbohydrate samples relative to that of glucose was calculated and expressed in terms of PAD responsiveness (see also text).

![Fig. 3 Changes in molecular-weight distributions of XGs mediated by a crude preparation of EXT.](image)

Ten μg of non-labeled XGs from *Vigna* (mol wt, 65 kDa) were incubated with 1 μg of a native (upper panel) or denatured (lower panel) crude preparation of enzyme derived from the apoplast of *Vigna* epicotyls in 10 μl of 0.2 M sodium acetate, pH 5.8 for 1 h at 25°C. After the reaction, the reaction mixture was analyzed by GPC under the conditions described in the legend to Fig. 3. The width halfway to the top of each peak of polymer was measured, expressed in ml and defined as the peak width. Arrow a indicates monosaccharide peak.

![Fig. 4 Changes in the distribution of molecular weights of XGs mediated by a purified EXT.](image)

Ten μg of non-labeled XG from *Vigna* (mol wt, 65 kDa) were incubated with 40 ng of purified EXT derived from the apoplast of *Vigna* epicotyls for 1 h under the conditions described in the legend to Fig. 3. Products of the reaction were analyzed by GPC under the conditions described in the legend to Fig. 1. Notice the reduced size of the peak of monosaccharides, indicated by arrow a (cf. Fig. 3).
Fig. 5 Relationship between the dose of enzyme and the increase in the peak width. Ten µg of XGs (mol wt, 65 kDa) were incubated with various amounts of crude preparation of apoplastic enzyme for 30 min under the conditions described in the legend to Fig. 3. After the products of the reaction had been fractionated by GPC, increases in the widths of peaks of XGs were measured and expressed in peak width units by the procedure described in Results and Discussion.

with the peak broadening mediated by EXT.

To quantify the degree of broadening we measured the peak width halfway to the top of the chromatogram and expressed the width in terms of the elution volume (in ml; Fig. 3). In an ideal normal distribution curve, the peak width halfway to the top of the peak is a function of its standard deviation (the peak width = 2.35 x standard deviation). Thus, the peak width provides an appropriate measure of the pattern of distribution of material and is independent of the total area of the peak. In the present study, we have defined one unit of peak width as the elution volume that corresponds to a log₁₀ (mol wt) of 0.301 on the ordinate in Figure 1. We employed the value 0.301 (= log₁₀ 2), because it corresponds to a two-fold difference in molecular weight. When we use 50-kDa XG, for example, as the substrate, one unit of peak width is calculated to be 1.07 ml from the diagram in Figure 1.

The EXT-mediated increase in the peak width was proportional to the dose of enzyme between 0 to 0.5 units (Fig. 5). Figure 6 shows that the peak width increased linearly during the first 30 min of the enzymatic reaction. These data show that peak broadening, as expressed in these units, is an appropriate measure of the transglycosylation reaction mediated by EXT.

Transfer of label—We used PA-XG as the acceptor molecule to quantify and characterize the transglycosylation reaction. PA-XG was prepared by tagging the reducing ends of XG molecules with a pyridylamino group, which is fluorescent. A mixture of 20 pmole of 500-kDa non-labeled XG (10 µg) and 200 pmole of 10-kDa PA-XG (2 µg) was used as the substrate for the enzyme reaction. These two substrates were resolved by GPC and detected by PAD (peaks a and b in Fig. 7). Transfer of XG segments from non-labeled XGs (donor) to PA-XGs (acceptor) was assessed by detection of high-molecular-weight fluorescent XGs by GPC (peak A in Fig. 7). The result clearly indicates that EXT mediates transfer of a high-molecular-weight XG segment from the 500-kDa XG molecule to the 10-kDa PA-

Fig. 6 Changes in peak width as a function of incubation time. Ten µg of XG were incubated with 1 µg of the crude preparation of enzyme for 1 h under the conditions described in the legend to Fig. 3 for various periods of time. The enzymatic activity was expressed in peak width units, as described in the legend to Fig. 5.

Fig. 7 GPC profiles of XG showing transfer of XG segments from non-labeled high-molecular-weight XG to low-molecular-weight PA-XG. A mixture of 10 µg of non-labeled XG from Vigna (mol wt, 500 kDa) (arrow a) and 2 µg of PA-XG (mol wt, 10 kDa) (arrows b and B) was incubated with 0 ng (upper panels), 10 ng (middle panels) or 30 ng (lower panels) of purified EXT for 1 h under the conditions described in the legend to Fig. 3. The products of the reaction were analyzed by GPC. Total XGs in the eluate were detected by a PAD, while PA-XG was detected by a fluorescence detector.
Detection of endo-xyloglucan transferase

XG molecule. Since the response of the fluorescence detector is proportional to the number of PA-XG molecules, the amount of high-molecular-weight PA-XG can be evaluated by measuring the area of peak A. The amount of high-molecular-weight PA-XG generated during a 1-h reaction was proportional to the amount of EXT present in the reaction mixture (Fig. 8). Thus, the amount of high-molecular-weight PA-XG is also a suitable measure of EXT activity.

Ten ng of EXT caused production of 22.4 pmole of high-molecular-weight PA-XG during incubation at 25°C for 1 h (Fig. 8). The initial reaction mixture contained 20 pmole of 500-kDa XG and 200 pmole of 10-kDa PA-XG. Accordingly, generation of 20 pmole of the high-molecular-weight PA-XG indicates that each 500-kDa XG molecule has undergone transglycosylation reaction 1.12 times, on average, during the incubation.

Investigations of the substrate specificity of EXT showed that the donor substrate activity depends on the molecular weight of donor substrates. Reactions catalyzed by EXT occur more rapidly when donor substrates with higher molecular weight are used (Nishitani and Tominaga 1992). Little or no transglycosylation is observed when XGs of less than 10 kDa are used as donor substrates. However, XG oligomers as small as 1 kDa can act as acceptors in the transglycosylation reaction. In the present study, no PA-XG smaller than 10 kDa was produced during the enzymatic reaction, as shown in Figure 7, indicating that EXT did not mediate the transfer of segments from 10-kDa XG to 500-kDa XG. This result is consistent with the substrate specificity of EXT (Nishitani and Tominaga 1992).

To examine the relationship between the rate of production the high molecular weight PA-XGs and the degree of peak broadening, as expressed in peak width units, the two experiments were conducted under the same conditions. Figure 9 shows the linear relationship between the two parameters, an indication that both parameters represent the transferase activity appropriately.

In conclusion, the results of the present study indicate that the activity of EXT is easily detected and quantified either (1) by measuring the distribution of molecular weights of XGs by GPC in a system equipped with a PAD or (2) by measuring changes in molecular weights of the PA-XG by GPC in a system equipped with a fluorescence detector as well as a PAD. Application of these new procedures has made it possible to detect and quantify EXT activity in 20 min without using any radioactivity. These procedures have been successfully applied for purification of EXT for the first time (Nishitani and Tominaga 1992) and also for characterization of the mode of action of this enzyme. With other polysaccharides and their pyridylamino derivatives as substrates, this new method offers the opportunity for a new approach to the search for unknown transglycosylases that may be involved in the construction and modification of the architecture of the plant cell wall.

Fig. 8 Relationship between dose of EXT and production of high-molecular-weight PA-XG. A mixture of 10 μg of non-labeled XG from Vigna (mol wt, 500 kDa) and 2 μg of PA-XG (mol wt, 10 kDa) was incubated with various amounts of purified EXT for 1 h under the conditions described in the legend to Fig. 3. The products of the reaction were analyzed by GPC, and the amount of high-molecular-weight PA-XG was calculated from the area (cf. peak A in Fig. 7) and expressed in pmole as a function of the dose of enzyme.

Fig. 9 Relationship between the production of high-molecular-weight PA-XG and the increase in the peak width. A mixture of 10 μg of non-labeled XG from Vigna (mol wt, 500 kDa) and 2 μg of PA-XG (mol wt, 10 kDa) was incubated with various amounts of purified EXT for 30 min under the conditions described in the legend to Fig. 3. The amount of high-molecular-weight PA-XG generated during the enzyme reaction was determined by the procedure described in the legend to Fig. 8. For measurement of the increase in the peak width, 10 μg of XGs from Vigna (mol wt, 65 kDa) was incubated with various amounts of purified EXT for 30 min under the same conditions described in the legend to Fig. 3.
The author wish to thank Professor Susumu Hizukuri and Dr. Yasuhito Takeda (Kagoshima University) for providing pullulans. This work was supported by a Grant-in-Aid for Scientific Research (no. 03804055) from the Ministry of Education, Science and Culture, Japan.

References


(Received February 28, 1992; Accepted September 28, 1992)