The Role of Wall Ca\textsuperscript{2+} in the Regulation of Wall Extensibility During the Acid-induced Extension of Soybean Hypocotyl Cell Walls

Naofumi Ezaki\textsuperscript{1}, Nobuo Kido, Koji Takahashi and Kiyoshi Katou

Division of Biological Science, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8602 Japan

We examined the acid-facilitated yielding properties of cell walls of soybean hypocotyls and the effects of Ca\textsuperscript{2+} upon the properties by stress–strain analyses using glycerinated hollow cylinders (GHCs) from the elongating regions of the hypocotyls. Stress–extension rate curves of native GHCs showed characteristic changes with pH, all indicating the existence of yield threshold tension (\(y\)) as well as wall extensibility (\(\phi\)), i.e. a downward shift of \(y\) and an increase in \(\phi\) with wall acidification. The acid-induced downward shift of \(y\) was inhibited by boiling of GHCs. In contrast, a considerable increase in \(\phi\) with acidification remained even after boiling. This indicates that \(\phi\) consists of two components, i.e. heat-sensitive and heat-resistant, both being pH sensitive. A Ca\textsuperscript{2+} chelator (Quin 2) dramatically increased \(\phi\) at a neutral pH. Subsequent addition of Ca\textsuperscript{2+} or ruthenium red suppressed the chelator-induced increase in \(\phi\). These findings suggest that wall Ca\textsuperscript{2+} plays an important role in the regulation of wall extensibility during the acid-induced wall extension by reacting with carboxyl groups of wall pectin.

Keywords: Glycine max — Pectin — Stress–strain properties — Wall Ca\textsuperscript{2+} — Wall extension.

Abbreviations: EGTA, ethylene glycol bis (\(\beta\)-aminoethyl ether)\textsubscript{N,N,N',N'-tetraacetic acid; GHC, glycerinated hollow cylinder of hypocotyl segment; \(\phi\), wall extensibility in vivo; \(\phi\), wall extensibility in vitro; Quin 2, 8-amino-2-(2-amino-5-methylphenoxymethyl)-6-methoxyquinoline-\(\textsubscript{N,N,N',N'}\)-tetraacetic acid; RR, ruthenium red; \(Y\), yield threshold pressure; \(y\), yield threshold tension.

Introduction

Extension of the cell wall is essential for elongation growth for plants. In steady-state growth, this process is formulated with a well-known empirical equation (Lockhart 1965), \(v = \Phi(P - Y)\), where \(v\) is the relative growth rate, \(\Phi\) is wall extensibility, \(P\) is turgor and \(Y\) is the yield threshold pressure. Auxin promotes extension of the cell wall by increasing wall extensibility (Masuda 1990, Cosgrove 2000, Hager 2003). According to the acid growth theory, auxin-induced acidification of the cell wall due to the activation of the plasma membrane proton pumps increases wall extensibility resulting in rapid cell elongation growth (Hager et al. 1971, Rayle and Cleland 1977). Expansin is a pH-sensitive wall-loosening factor originally isolated from cucumber hypocotyls (McQueen-Mason et al. 1992, Shcherban et al. 1995). Expansin, widely distributed in plants, rapidly promotes cell creep under an acidic condition by increasing wall extensibility due to weakening of hydrogen bonding between hemicellulose and cellulose polymers (McQueen-Mason and Cosgrove 1994, McQueen-Mason 1997, Cosgrove 2000).

Although auxin was considered to have no effect on effective turgor (\(P - Y\)) (Green and Cummins 1974, Cleland 1977, Cosgrove 1985), it was shown to increase the effective turgor; \(Y\) was shifted downward without changing \(P\) (Nakahori et al. 1991, Maruyama and Boyer 1994). In cowpea hypocotyl segments, \(\Phi\) increased and \(Y\) shifted downward upon acidification of the cell wall (Mizuno et al. 1993), which was favorable to the acid-induced growth. Furthermore, acid-induced changes in these parameters were also observed in an in vitro system, i.e. in glycerinated hollow cylinders (GHCs) from the elongating regions of cowpea hypocotyls (Okamoto and Okamoto 1994). Okamoto and Okamoto (1995) suggested the participation of two kinds of wall proteins in the regulation of both wall extensibility (\(\phi\)) and yield threshold tension (\(y\)). A \(\gamma\)-adjusting protein named yieldin was isolated from cowpea hypocotyls and cloned as chitinase-like protein (Okamoto-Nakazato et al. 2000a, Okamoto-Nakazato et al. 2000b), but it has not been reported in other plants. There are also only a few reports of the pH-dependent adjustment of \(y\) of the cell wall in vitro in other plants (Taguchi et al. 1999).

The cleavage of calcium bridges between pectic carboxyl groups in the cell wall has been supposed to play important roles in wall extension (Bennet-Clark 1956, Tagawa and Bonner 1957, Soll and Böttger 1982). High concentrations of Ca\textsuperscript{2+} inhibit cell elongation of Avena coleoptiles (Cool and Bonner 1957) and EDTA promotes elongation growth of lupin hypocotyls (Weinstein 1956). As Ca\textsuperscript{2+} binding to uronic acids is easily exchanged for H\textsuperscript{+} (Sentenac and Grignon 1981), this exchange reaction may take part in the acid-induced extension of the cell wall. Virk and Cleland (1988) showed a direct correlation between the amounts of removed wall Ca\textsuperscript{2+} and the increase in plastic extensibility of the cell wall. However, they concluded that breakage of Ca\textsuperscript{2+} bridges is not a major mechanism of the acid-facilitated cell wall loosening because Ca\textsuperscript{2+} chelators caused only limited facilitated creep as compared...
Acid-induced wall extension and Ca\(^{2+}\) in soybean

with acid (Virk and Cleland 1990). Their creep assay (i.e., measurement of wall extension under a constant tension) is suitable for studies on wall extension during plant elongation growth, as turgor remains unchanged during auxin-induced growth promotion (Cosgrove and Cleland 1983, Nakahori et al. 1991, Maruyama and Boyer 1994). With creep assay, however, one cannot recognize whether wall loosening is caused by the changes of \(\phi\) and/or \(y\). Therefore, the contribution of wall Ca\(^{2+}\) to the acid-facilitated wall loosening needs to be re-evaluated taking pH-dependent changes in two wall yielding parameters into consideration.

In this study, we examined yielding properties of soybean hypocotyl cell walls, by stress–strain analysis using GHCs, and the effects of Ca\(^{2+}\) on the properties.

**Results**

Yielding properties of GHCs from elongating regions of soybean hypocotyls and their pH dependencies

Wall yielding properties of soybean GHCs were studied under tension increased stepwise by weight loading. Fig. 1A shows the stress–extension rate curves of native GHCs at pH 6.0–4.0. The rate of wall extension characteristically increased with an increase in tension depending on pH. Under a low range of tension, an increase in the extension rate was low (low-rate extension). However, it became high and markedly sensitive to pH after the extension rate exceeded 0.5–1.0% h\(^{-1}\) (high-rate extension). The stress–extension rate curve apparently had a yield threshold at each pH, indicating the existence of a yield threshold tension \((y)\) in soybean hypocotyl cell wall. We estimated \(y\) and wall extensibility \((\phi)\) as the x-axis intercept and the slope of the regression line for the high-rate extension, respectively (Fig. 1A).

Fig. 1B shows the pH dependency of stress–extension rate curves of boiled GHCs. Although the boiled GHCs were too

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**Fig. 1** Stress–extension rate curves of native (A) and boiled (B) GHCs from soybean hypocotyls at pH 6.0 (circle), 5.5 (square), 5.0 (diamond) and 4.0 (triangle). Solid and open symbols represent low-rate and high-rate extension, respectively. The line on each curve is the regression line for the high-rate extension. The slope and x-axis intercept of the regression line are \(\phi\) and \(y\), respectively. Tension was increased stepwise. Typical data from several experiments \((n \geq 6)\) under each pH condition are shown.

**Fig. 2** pH profiles for wall extensibility (A) and yield threshold tension (B) of native and boiled GHCs from soybean hypocotyls. Open and solid symbols represent the values in native and boiled GHCs, respectively. Wall extensibility and yield threshold tension were calculated from stress–extension rate curves of native and boiled GHCs at each pH. A dotted line and dashed line are the regression lines fitted by the least squares method. The data are means ± SD \((n \geq 6)\). In the pH range 5.5–4.5, there are significant differences in \(\phi\) between native and boiled GHCs \((P < 0.01)\). In the pH range 5.5–3.5, there are significant differences in \(y\) between native and boiled GHCs \((P < 0.001)\).
Effects of Ca\textsuperscript{2+} and a chelating agent on yielding properties of GHCs

Exogenous Ca\textsuperscript{2+} markedly inhibited wall extension by not affecting \( y \) but significantly reducing \( \phi \). Ca\textsuperscript{2+} at 10 mM, however, slightly shifted \( y \) upward. Table 1 summarizes the effects of exogenous Ca\textsuperscript{2+} on \( \phi \) and \( y \) at pH 5.0.

Table 1  Effects of exogenous Ca\textsuperscript{2+} on the mechanical parameters of native and boiled GHCs from soybean hypocotyls

<table>
<thead>
<tr>
<th>Samples</th>
<th>Perfusion conditions</th>
<th>( n )</th>
<th>( \phi ) (% h\textsuperscript{-1} gf\textsuperscript{-1})</th>
<th>( y ) (gf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>pH 5.0</td>
<td>6</td>
<td>0.201 ± 0.027&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.1 ± 7.8&lt;sup&gt;xy&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 5.0 + 1 mM CaCl\textsubscript{2}</td>
<td>5</td>
<td>0.106 ± 0.018&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.4 ± 3.6&lt;sup&gt;y&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>pH 5.0 + 3 mM CaCl\textsubscript{2}</td>
<td>5</td>
<td>0.087 ± 0.018</td>
<td>79.1 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>pH 5.0 + 10 mM CaCl\textsubscript{2}</td>
<td>5</td>
<td>0.034 ± 0.010</td>
<td>83.0 ± 7.6&lt;sup&gt;y&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Boiled, 20 s</td>
<td>pH 5.0</td>
<td>6</td>
<td>0.103 ± 0.017&lt;sup&gt;c&lt;/sup&gt;</td>
<td>109.6 ± 7.4&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH 5.0 + 10 mM CaCl\textsubscript{2}</td>
<td>6</td>
<td>0.045 ± 0.024&lt;sup&gt;c&lt;/sup&gt;</td>
<td>112.6 ± 8.8&lt;sup&gt;y&lt;/sup&gt;</td>
<td></td>
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Yielding properties of native and boiled GHCs were measured in buffer at pH 5.0 with or without CaCl\textsubscript{2} at 1–10 mM. The values are means ± SD and \( n \) is the number of experiments.

<sup>a,b,c</sup> The same letters are significantly different from each other (\( P < 0.001; \quad b \), \( P < 0.05 \)).

Fig. 3A shows the effects of removal of wall Ca\textsuperscript{2+} on yielding properties of GHCs. Pre-treatment of GHCs with 1 mM 8-amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-N,N,N',N'-tetraacetic acid (Quin 2), a Ca\textsuperscript{2+} chelator that can remove wall Ca\textsuperscript{2+} at pH 6.0 more effectively than ethylene glycol bis (\( \beta \)-aminoethyl)ether)-N,N',N,N'-tetraacetic acid (EGTA) with a little acidification of solution (Tsien et al. 1982, Rayle 1989, Virk and Cleland 1990), markedly increased the \( \phi \) at pH 6.0 (from 0.036 to 0.150% h\textsuperscript{-1} gf\textsuperscript{-1}), and simultaneously caused a considerable downward shift of \( y \). The increase in \( \phi \) was about 82% of the \( \phi \) value in untreated GHCs at pH 4.0 (0.183% h\textsuperscript{-1} gf\textsuperscript{-1}) (Table 2). At pH 4.0, little difference was found in \( \phi \) and \( y \) between Quin 2-treated and untreated GHCs. Even in boiled GHCs, the Quin 2-induced increase in \( \phi \) at pH 6.0 was found (Table 2). Subsequent addition of Ca\textsuperscript{2+} (0.01–0.10 mM) reduced the Quin 2-induced increase in \( \phi \) (Fig. 3B), but did not restore the shifted \( y \). Ruthenium red (RR), being different from previous reports in cucumber (McQueen-Mason et al. 1992) and cowpea (Okamoto and Okamoto 1995). At pH 6.0 and 3.5, no significant differences of \( \phi \) and \( y \) were found between native and boiled GHCs. In boiled GHC, the acid-induced downward shift of \( y \) was slight between pH 6.0 and 4.0 but sharp between pH 4.0 and 3.5 (Fig. 2B).
known to bind selectively to pectin (Sterling 1970), showed similar effects to Ca$^{2+}$ at 0.03 mM (Fig. 3C, Table 2). In 0.1 mM RR-treated GHCs, however, the high-rate extension was too low to estimate the correct $\gamma$ values. RR at 0.1 mM practically inhibited the high-rate extension.

**Comparison between Quin 2-induced and acid-induced creeps of GHCs**

Fig. 4A shows the time course of wall creep induced by pH 5.5 and Quin 2 under a load of 130 g. The estimated values of $\phi$ and $\gamma$ at pH 6.0 in Quin 2-pre-treated GHCs (0.150% h$^{-1}$ gf$^{-1}$ and 88 gf) were close to those at pH 5.5 in native GHCs (0.111% h$^{-1}$ gf$^{-1}$ and 82 gf). The Quin 2-induced creep was comparable with the acid-induced creep at least within 1.5 h (Fig. 4A). The Quin 2-induced creep was sustained over 3 h, but the acid-induced creep was sustained for only 2–2.5 h due to the ensuing breakage of GHCs.

Since Ca$^{2+}$ chelation with Quin 2 causes slight acidification of a solution (Rayle 1989), the Quin 2-induced creep of GHC might not be caused by removal of wall Ca$^{2+}$ but by wall acidification. In order to examine this possibility, the GHC under Quin 2-induced creep promotion was subjected to the pH 6.0 buffer without Quin 2 (Fig. 4B). There was no suppression of Quin 2-induced creep by this treatment, while the acid-induced creep (pH 5.5) was reversibly suppressed by pH 6.0 buffer.

**Discussion**

In soybean hypocotyl cell walls, yield threshold tension ($\gamma$) apparently exists (Fig. 1A) as in cowpea (Okamoto and Okamoto 1994). In cowpea, the yield threshold tension was estimated as the tension at which the regression line of the low-rate extension intersected the regression line of the high-rate extension. However, this is not really the $\gamma$ defined by the Lockhart equation of wall extension (Lockhart 1965). The rate of wall extension around this intersection often increased gradually and reached a high extension rate, suggesting gradual switching from the low-rate extension to the high-rate extension with an increase in tension. It is apparent that the wall yielding responsible for acid-induced and therefore auxin-induced growth is the high-rate extension. The $\gamma$ and $\phi$ which we estimated as the x-axis intercept and the slope of the regression line for the high-rate extension brought more comprehensible results. Our method of estimation is essentially the same as that applied for frozen-thawed segments of *Avena* coleoptiles (Cleland et al. 1987).
Acid-induced wall extension and Ca\(^{2+}\) in soybean

The almost linear change in wall extension because the native GHCs fit well with the pH-dependent regulation of 1991, Peters and Felle 1991). Thus the observed narrow pH of cell wall materials (Böttger et al. 1980, Mizuno and Katou 1991). At pH 4.0, it was difficult for the wall pH to be decreased from 6.0 to 5.0 because of the high buffering capacity of cell wall materials (Böttger et al. 1980, Mizuno and Katou 1991). Therefore, it is necessary to identify the pH-adjusting wall proteins from soybean hypocotyls.

The boiling effect on \(\phi\) suggested that the \(\phi\) regulation consisted of two processes, heat-sensitive and heat-resistant. The heat-sensitive component of \(\phi\) \(\phi_{\text{native}}\) minus \(\phi_{\text{boiled}}\) showed a bell-shaped change against pH (Fig. 5A), indicating that some wall proteins having maximum activity around pH 5.0 might aid the \(\phi\) of native cell walls in increasing sharply and linearly with a pH decrease from 6.0 to 5.0. The bell-shaped profile indicates that the activity of this wall protein is enzymatic. On the other hand, the linear increase in the heat-resistant component with pH decrease from 6.0 to 3.5 suggests that non-protein-mediated physicochemical reactions play a fundamental role in the pH-dependent regulation of \(\phi\) in soybean cell wall.

Removal of wall Ca\(^{2+}\) with Quin 2 promoted the creep of GHC comparable with the acid-induced promotion (pH 5.5) (Fig. 4A). Quin 2 irreversibly degrades the cell wall of Ca\(^{2+}\), while acid may solubilize and keep Ca\(^{2+}\) in the cell wall. Stress-strain analysis using the GHCs pre-treated with Quin 2 revealed that Quin 2-induced wall extension was mainly caused by the increase in \(\phi\) (Fig. 3A). In particular, the heat-resistant component of \(\phi\) is regulated by some wall Ca\(^{2+}\) because even in boiled GHC an increase in \(\phi\) was caused by Quin 2 treatment at pH 6.0 (Table 2). In addition, the fact that exogenous Ca\(^{2+}\) inhibited not \(\phi\) but only \(\phi\) suggests that Ca\(^{2+}\) is related to the regulation of \(\phi\) in soybean GHCs (Table 1). RR, a specific indicator for pectin, binds with a negative charge between each pectin monomer and its next adjacent neighbor (Sterling 1970). The RR-induced suppression of the Quin 2-increased \(\phi\) (Fig. 3C, Table 2) suggests that pectin reacts \(\phi\) dependently with endogenous Ca\(^{2+}\) and is involved in the acid-facilitated extension of the cell wall. Therefore, the cleavage of Ca\(^{2+}\) bridges between pectin molecules must play a key role in the pH-dependent regulation of \(\phi\) in the soybean cell wall.

Virk and Cleland (1990) concluded that the breakage of Ca\(^{2+}\) bridges is not a major mechanism of acid-facilitated wall loosening in soybean hypocotyl tissues because (i) Ca\(^{2+}\) chelator caused only a limited facilitated creep as compared with acid; and (ii) reversible acid-induced extension occurred even

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**Fig. 5** The heat-sensitive component of \(\phi\) and the suppression of \(\gamma\) shift by boiling at each pH. (A) The heat-sensitive component of \(\phi\) was calculated by subtracting \(\phi_{\text{boiled}}\) from \(\phi_{\text{native}}\) at each pH. (B) The degree of \(\gamma\) shift suppression by boiling was calculated by subtracting \(\gamma_{\text{native}}\) from \(\gamma_{\text{boiled}}\) at each pH.
Acid-induced wall extension and \( \text{Ca}^{2+} \) in soybean

after removal of almost all \( \text{Ca}^{2+} \) with Quin 2. In order to reconsider their conclusion, we simulated the facilitated creep reported by Virk and Cleland as the creep in a diagram shown in Fig. 6. Comparing the extension rates, it was found that the 20 g load which Virk and Cleland applied to bisected frozen–thawed sections in their creep assay corresponded to the 60 g load (a dotted line) in our stress–strain analysis using GHCs. Under this 60 g load, treatment with Quin 2 causes only a small increase in the creep rate at pH 6.0 because the applied load is considerably below the yield threshold tension (88 gf) despite a significant increase in \( \phi \). Subsequent acidification to pH 4.0 causes rapid wall creeping due to a sufficient \( y \) shift to 52 gf below the applied load. The acid-induced shift of \( y \) which takes place irrespective of chelator pre-treatment is thought to result in reversible acid-induced extension after \( \text{Ca}^{2+} \) removal. Thus, our results are essentially the same as those of Virk and Cleland (1990). However, our results indicate that the facilitated creep due to the Quin 2-induced increase in \( \phi \) must occur if an applied load is sufficiently large over the \( y \), e.g. 110 g in Fig. 6 (a dashed line). Actually, Quin 2 caused facilitated creep at pH 6.0 comparable with the acid-facilitated creep at pH 5.5 (from 115 to 82 gf). The effect of \( \text{Ca}^{2+} \) removal might modulate the activity of \( y \)-regulating proteins, because Quin2 hardly affected the \( y \) of boiled GHCs (Table 2). Another possibility is the chelator effects on wall polymers. Rhamnogalacturonan II (RG-II), a constituent of pectic polysaccharides, exists as a dimer cross-linked by a borate diol ester (B–RG-II complex) in the primary cell wall (Ishii and Matsunaga 1996, O’Neill et al. 1996). Since \( \text{Ca}^{2+} \) removal with a chelating agent is known to convert the B–RG-II complex to monomeric RG-II (Kobayashi et al. 1999), the degradation of the B–RG-II complex might cause a downward shift of \( y \). However, the effect of \( \text{Ca}^{2+} \) removal on \( y \) is uncertain because much remains unknown about the molecular nature of \( y \) regulation.

Materials and Methods

Plant materials

Soybean (Glycine max Merr. cv. Enrei, Asahi-noen Co., Aichi, Japan) seeds from a production lot of the same harvest year were immersed for 1 h in tap water at 25\(^\circ\)C and planted in wet sand. Seedlings grown for 3 d at 25\(^\circ\)C in the dark and with hypocotyls 6–7 cm long were used for all experiments. Hypocotyl segments 15 mm long were excised from the elongating region beneath the hook. GHCs for stress–strain experiments were prepared by essentially the same procedure as reported for cowpea hypocotyls (Okamoto and Okamoto 1994). Excised segments were bored with a stainless steel pipe 1.5 mm diameter, infiltrated with 50% glycerol aqueous solution under 1 kPa for 30 min, and stored in 50% glycerol at −20\(^\circ\)C until use. GHCs were prepared three times within 2 weeks. Each lot consists of 50–100 GHCs. The GHCs for experiments were randomly selected from three preparation lots.

For heat inactivation, GHCs were dipped in boiling water for 15–20 s before use. This treatment is known to inactivate cucumber
expansins (McQueen-Mason et al. 1992), and to be much more drastic than the heat treatments for inactivation of yieldin and putative \( \phi \) protein in cowpea GHCs (Okamoto and Okamoto 1995).

**Measurement of yield properties of GHCs**

The yield properties of soybean GHCs were analyzed with an extensometer, basically the same as that used for cowpea (Okamoto and Okamoto 1994) with slight modifications. A GHC was fixed to polycarbonate flanges with cyanoacrylate glue, and mounted on the extensometer leaving 5 mm for extension. The GHC was dipped in a buffer solution (10 mM MES-NaOH for pH 6.0 and 10 mM dimethylglutaric acid-NaOH for pH 5.5–3.5) and perfused with the same buffer through its central hollow during measurements. Testing reagents were added to the buffer when necessary. The extension of the GHC was measured with a differential transformer (DTD-3; Seiyu Electronics Co., Kawasaki, Japan) and recorded with a computer-aided data acquisition and analyzing system (PowerLab 2/20; ADInstruments Pty Ltd, Castle Hill, Australia). The rate of extension was calculated simultaneously by differentiating DTD output, and displayed.

**Tensional stress was increased stepwise at intervals of 20–30 min by weight loading. Just after a weight loading, a large transient increase in the rate was always observed, but the rate reached a steady state within 10–15 min (Fig. 7).** The extension rate under each load was estimated 15–30 min after a weight loading. Under a low range of applied loads, the extension rate gradually increased with an increase in the applied load. When an applied load exceeded the load that caused the extension of about 1.0% h\(^{-1} \), a marked increase in the extension rate occurred with further loading, finally resulting in breakage of the GHC. Just before the breakage, the extension always became unstable within 20 min after loading, as shown in Fig. 7 (in the case of a 125 g load). We regarded this unstable extension as an indication of the GHC being broken and discarded. Unstable extension was often observed when the extension rate exceeded 3.0–3.5% h\(^{-1} \). Therefore, we regarded the rate of 3.5% h\(^{-1} \) as the upper limit of the high-rate extension. Regression lines of the high-rate extension were fitted for 3–6 points of the extension rates below 3.5% h\(^{-1} \) by the least squares method. All extensometric procedures were carried out at 25 ± 1°C and repeated at least five times.

**Acknowledgments**

This work is supported in part by a grant from Daiko Foundation (N.K.) and by a Grant-in-Aid for Scientific Research (C) (No. 14540592) from the Ministry of Education, Culture, Sports, Science, and Technology.

**References**


(Received May 30, 2005; Accepted September 2, 2005)