Plant Cell Physiol. 47(11): 1520–1529 (2006)
doi:10.1093/pcp/pcl017, available online at www.pcp.oxfordjournals.org

Wall-Yielding Properties of Cell Walls from Elongating Cucumber Hypocotyls in Relation to the Action of Expansin

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The wall-yielding properties of cell walls were examined using frozen–thawed and pressed segments (FTPs) obtained from the elongation zones of cucumber hypocotyls with a newly developed programmable creep meter. The rate of wall extension characteristically changed depending on both tension and pH. By treatment of the FTPs with acid, the yield tension (y) was shifted downward and the extensibility (ϕ) was increased. However, the downward shift of y was greatly suppressed and the increase in ϕ was partly inhibited in boiled FTPs. The boiled FTPs reconstituted with expansin fully recovered the acid-induced downward y shift as well as the increase in ϕ. Even under the tension below y, wall extension took place pH dependently. Such extension was markedly slower (low-rate extension) than that under the tension above y (high-rate extension). At a higher concentration (8 M), urea markedly inhibited the creep ascribable to the inhibition of the acid-induced downward y shift and increase in ϕ. Moderate concentrations (2 M) of urea promoted wall creep pH dependently. The promotion was equivalent to a 0.5 decrease in pH. The promotion of creep by 2 M urea was observed in boiled FTPs reconstituted with expansin but not in boiled FTPs. These findings indicated that the acid-facilitated creep was controlled by y as well as ϕ in cucumber cell walls. However, y and ϕ might be inseparable and mutually related parameters because the curve of the stress extension rate (SER) showed a gradual change from the low-rate extension to the high-rate extension. Expansin played a role in pH-dependent regulation of both y and ϕ. The physiological meaning of the pH-dependent regulation of wall creep under different creep tensions is also discussed with reference to a performance chart obtained from the SER curves.

Keywords: Acid-facilitated wall creep — *Cucumis sativus* — Expansin — Programmable creep meter — Wall extensibility — Yield stress.

Abbreviations: ϕ, wall extensibility (% $gf^{-1}\,h^{-1}$); FTP, segment of frozen–thawed and pressed hypocotyl; GHC, glycerinated hollow cylinder of hypocotyl; PCM, programmable creep meter; SER, stress extension rate; v, relative rate of wall extension (% h^{-1}); y, yield tension (gf).

Introduction

Irreversible wall extension is one of the key processes in plant elongation growth. The rate of wall extension in vivo has been analyzed in terms of the rheological equation $v = \Phi\ (P - Y)$ (Lockhart 1965, Ray et al. 1972), where v is the relative growth rate, Φ is the relative extensibility, P is turgor and Y is the yield pressure. Auxin promotes wall extension by simultaneously increasing Φ and effective turgor $(P - Y)$ (Nakahori et al. 1991, Maruyama and Boyer 1994) while keeping turgor P unchanged (Cosgrove and Cleland 1983, Nakahori et al. 1991). Thus, Y as well as Φ plays an important role in the regulation of plant elongation growth induced by auxin and/or acids (Nakahori et al. 1991, Katou and Okamoto 1992, Mizuno et al. 1993). These relationships were also found in the stress–strain analysis of the extension of isolated cell walls using glycerinated hollow cylinders (GHCs) of hypocoytl segments (Okamoto and Okamoto 1994, Okamoto-Nakazato et al. 2000a, Okamoto-Nakazato et al. 2000b, Ezaki et al. 2005). The acid-facilitated extension of isolated cell walls is regulated by both y and ϕ. In a steady state,

$$v = (1/l)(\mathrm{d}l/\mathrm{d}t) = \phi(\tau - y) \text{ for } \tau > y \qquad (1)$$

where v is the relative extension rate, l is the length of a wall specimen from growing stems, ϕ is the wall extensibility that depends on pH, τ is the applied tension and y is the yield tension that depends on pH. The stress extension rate (SER) curves of isolated cell walls of cowpea and soybean clearly indicated that the extension of the cell wall was promoted by a downward shift of y as well as by an increase in ϕ.

Two wall proteins, expansin and yieldin, are known to be involved in acid-facilitated wall extension. Auxin induces elongation of isolated stems keeping turgor unchanged (Cosgrove and Cleland 1983, Nakahori et al. 1991, Maruyama and Boyer 1994). No significant differences in turgor in vivo were reported between cells before elongation and cells during elongation (Pritchard et al. 1987, Rich and Tomos 1988). Therefore, the wall extension relevant to stem elongation is creep where creep is

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gradual deformation of plastic solid under a constant stress. Expansin was isolated from the cell walls of growing cucumber hypocotyls by creep assays using frozen–thawed and pressed segments (FTPs) (McQueen-Mason et al. 1992). Two genomic families of expansin with a wide distribution have been found (Cosgrove 2000, Cosgrove et al. 2002). Expansin was the first wall protein that promoted the acid-facilitated wall creep in vitro. It was proposed that expansin enhanced wall creep in acidic conditions by disrupting hydrogen bonding between cellulose and hemicellulose microfibrils (McQueen-Mason and Cosgrove 1994, Whitney et al. 2000). This mechanism suggests that expansin enhances wall extension by increasing the wall extensibility in acidic conditions. However, the rate of wall creep is regulated by either ϕ or y (see Equation 1). Therefore, the effects of expansin on these two rheological parameters in the regulation of wall extension are very interesting. Yieldin, a chitinase-like wall protein isolated from cowpea hypocotyls by stress–strain analysis with stepwise weight loading using GHCs, was found to enhance wall extension by causing a downward shift of y under acidic conditions without affecting ϕ (Okamoto-Nakazato et al. 2000a, b). Yieldin is located in the apoplastic walls of the peripheral cortex of the elongation zone and the hook of cowpea hypocotyls (Okamoto-Nakazato et al. 2001). However, wall proteins reacting with anti-yieldin antibody have not been found in the cell walls except for *Vigna* species (unpublished).

In this study, we examined the changes in the yielding parameters (ϕ and y) of isolated cucumber cell walls and the roles of expansin in the regulation of these parameters in relation to the control of plant elongation growth using FTPs. For the SER analysis, a newly developed programmable creep meter (PCM) was used.

Results

Measurements of the SER characteristics of isolated cell walls with the PCM

The SER curves of cucumber FTPs measured by the ramped sweep method using the PCM were carefully compared with those measured by the stepwise method using the PCM and/or manual extensiometer, and characterized. Fig. 1A and B shows the SER characteristics of cucumber FTPs measured at pH 4.5 with the PCM by a stepwise increase of tension (an increment of 2 gf and a duration of 30 min a step). The wall length increased non-linearly with a stepwise increase in tension. A large transient increase in the extension rate (v) was observed at every step of increase in tension. Thereafter, v reached nearly a steady state within about 30 min. The SER curves could be estimated from the steady v, and a regression line to the high-rate extension in the curves could be

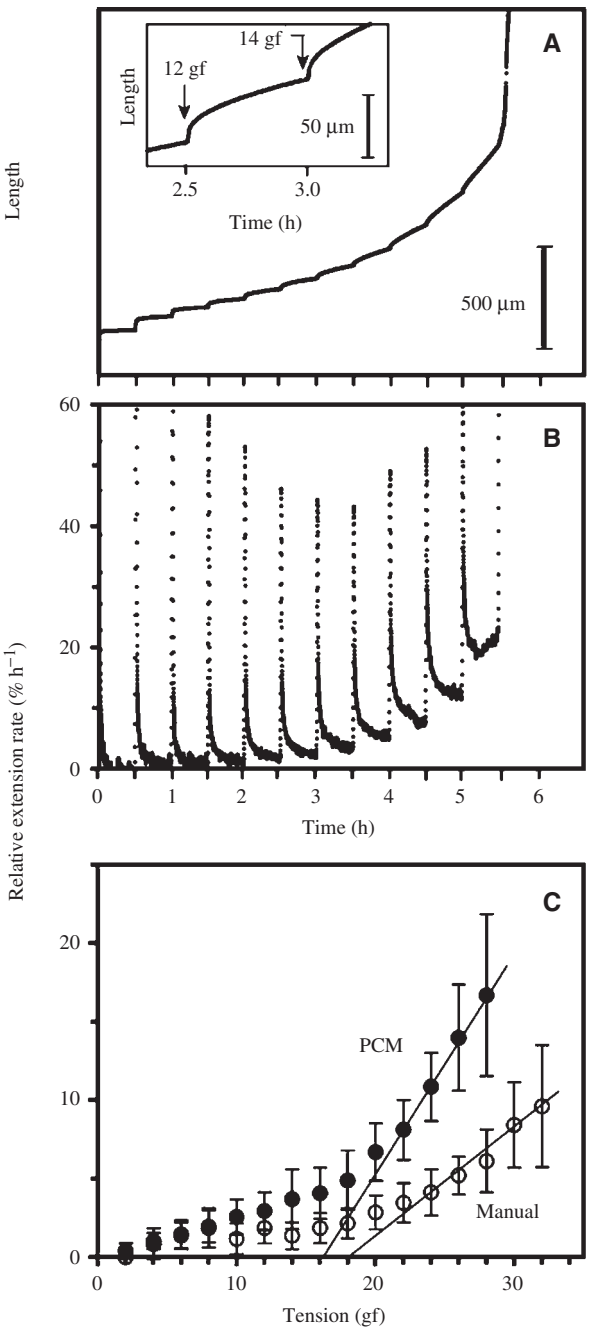

Fig. 1 The SER characteristics of frozen–thawed segments from the elongation zone of cucumber hypocotyls measured with PCM by the stepwise method. Typical wall extension (A) and the extension rate (B) of a native FTP were measured at pH 4.5 by a stepwise increase in tension (a 2 gf increase every 30 min). The bar in (A) represents 500 μm. The inset shows a magnified trace of the wall extension. The bar in the inset represents 50 μm. (C) SER curves were estimated from the steady rates of wall extension under each tension. Filled circles represent the data measured with the PCM, and open circles the data measured by manual extensiometry. Lines are the regression lines to the high-rate extension of the SER curves. Circles and bars in C indicate the means of five independent experiments and the SD.

approximated (Fig. 1C). As discussed previously (Ezaki et al. 2005), the values of ϕ and y were estimated from the slopes and x-intercepts of the regression lines, respectively. The present SER curve evidently indicated that cucumber cell walls also had the y, as in the case of cowpea and soybean. The ϕ estimated with the PCM was always greater than that estimated by the manual extensiometry (see 'PCM' and 'Manual' in Fig. 1C). However the essential features of the SER curves obtained using the PCM and manual extensiometer were not different. Both curves had the low-rate extension and the high-rate extension regions, and y showed similar dependence on pH.

Fig. 2A shows examples of the SER traces of cucumber FTPs measured by the ramped sweep method ($0.1\,gf\,min^{-1}$) at pH 4.5 using the PCM. A transient increase in v was always observed at the beginning of the tension sweep followed by the usual dependence of v on the tension. The curves were very much affected by the rate of ramped sweep. A higher sweep rate made the initial transient larger and the ensuing increase in the extension rate steeper (data not shown). In cucumber cell walls, the SER curves at a sweep rate of $<0.1\,gf\,min^{-1}$ were found to be nearly parallel to those observed by stepwise loading except the initial transient (Fig. 2B and 2C). A pre-ramped sweep from 0.5 to 2.5 gf at the rate of $0.2\,gf\,min^{-1}$ (twice as fast as that of the measuring sweep) was effective to minimize the effects of the initial transient on the SER curve (trace b in Fig. 2A). The SER curve between the tension of 6 gf and the rupture tension (i.e. between arrow 1 and arrow 2 in Fig. 2B) was essentially the same as that estimated by the stepwise method. This SER curve was approximated with two regression lines (L1 and L2 in Fig. 2B). The y-intercepts of the low-rate regression line (Fig. 2B) could be regarded as the origin of the SER curve measured at a given sweep rate, i.e. the point of no wall extension under zero tension. The values of the y-intercepts (h in Fig. 2B) were not different between pH 4.5 and 6.8 under a given sweep rate. Therefore, the SER curves obtained by ramped sweep could be regarded as being equivalent to the SER curves obtained by stepwise loading if the curves were corrected by deleting the initial transient and by subtracting an increase of the y-intercept (h in Fig. 2B). The values of ϕ and y for cucumber FTPs were estimated to be $1.29 \pm 0.23\%\ gf^{-1}\,h^{-1}$ and 15.8 ± 0.5 gf at pH 4.5, respectively.

Dependence of the yielding properties of cucumber cell walls on pH

The SER properties of cucumber cell walls could be measured more precisely by the newly developed method of ramped sweep using the PCM. The dependence of the yielding properties of cucumber FTPs on pH (6.8–3.5) was examined. Fig. 3A shows the pH dependence of the

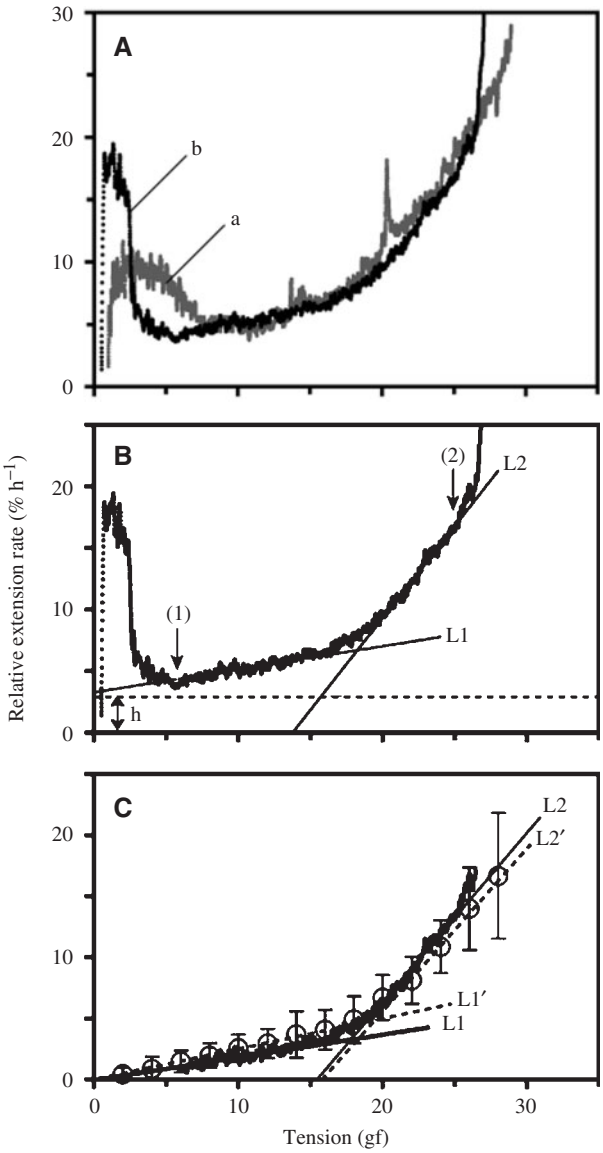

Fig. 2 Measurements of SER characteristics of cucumber cell walls with the PCM by the ramped sweep method. (A) Typical traces of the relative wall extension rate measured by the ramped sweep method at pH 4.5. Tension was applied by two programmed sweeps: (a) the tension was linearly increased from 0.5 gf to the rupture tension at the rate of $0.1\,gf\,min^{-1}$ and (b) the tension was programmed to increase initially at the rate of $0.2\,gf\,min^{-1}$ (from 0.5 to 2.5 gf) and then at the rate of $0.1\,gf\,min^{-1}$ (from 2.5 gf to the rupture tension). (B) Characterization of the SER curve measured by the ramped sweep method (produced from curve b in A). The two indicated lines (L1 and L2) are the regression lines to the low-rate extension and the high-rate extension, respectively. The 'h' represents the y intercept of L1. (C) The SER curves measured by the ramped sweep method (a continuous trace) and by the stepwise method (open circles). Solid lines (L1 and L2) and broken lines (L1′ and L2′) are the regression lines to the SER curves by ramped sweep and stepwise loading, respectively.

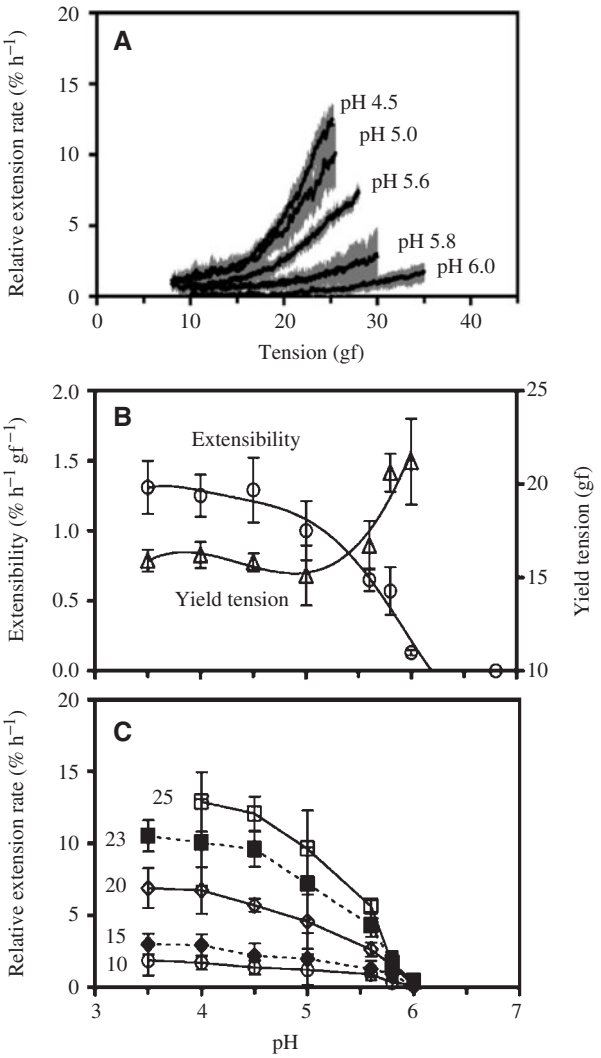

Fig. 3 The pH dependence of the SER characteristics of cucumber cell walls. (A) SER curves of the cucumber FTPs at pH 4.5, 5.0, 5.6, 5.8 and 6.0. Each curve was drawn as the averaged curve from the data of five independent experiments. The gray zone behind each curve indicates the SD. (B) pH profiles for the wall extensibility ϕ (open circles) and the yield tension *y* (open triangles), which were calculated from the above SER curves. (C) pH dependence of the creep rate of cucumber cell walls under different tensions (10, 15, 20, 23 and 25 gf).

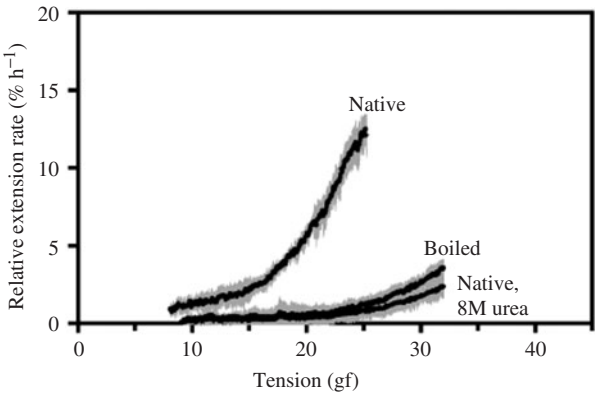

Fig. 4 Effects of boiling and 8 M urea on the SER characteristics of cucumber cell walls. All curves were measured by the ramped sweep method. The curves designated as 'Native', 'Native, 8M urea' and 'Boiled' were measured for native cucumber FTPs at pH 4.5, those in the presence of 8 M urea, and boiled FTPs at pH 4.5, respectively. The FTPs were boiled by dipping in boiling water for 15 s. Each curve was drawn as the averaged curve from the data of five independent experiments. The gray zone behind each curve indicates the SD.

corrected SER curves of native FTPs that were drawn from averaged data sets of five independent measurements at each pH. All the SER curves showed a similar pattern of dependence on the applied tension, i.e. the curve consisted of three distinct regions: the low-rate extension, the intermediate extension and the high-rate extension. The high-rate extension was always observed after the tension exceeded a critical value, indicating the existence of *y*. As the pH decreased, ϕ increased but *y* shifted downward at the same time (Fig. 3B). The pH dependence of the rate of extension varied with the applied tension (Fig. 3C). The rate of extension was very low and only slightly dependent on pH when the tension was <15 gf. However, it was distinctly high and much more dependent on pH under a tension of >20 gf. Under a tension between 20 and 23 gf, the rate of extension changed linearly with the change in wall pH between 5.6 and 4.5.

Effects of boiling and high concentrations (8 M) of urea on the SER characteristics

The heat treatments by dipping FTPs in boiling water for 15 s caused marked effects on the SER characteristics, as shown in Fig. 4. The boiling treatment fully inhibited the acid-induced *y* shift, although approximately 30% of the pH-sensitive ϕ remained even after the boiling. Boiling and 8 M urea had similar effects on the yielding properties of cell walls (Fig. 4, Table 1).

Effects of expansin on the yielding properties of cucumber cell walls

Expansin was isolated from the elongation zone of etiolated cucumber hypocotyls, and the effects of expansin on the SER properties were analyzed by reconstitution studies. Wall proteins were extracted with 1 M NaCl from the elongation zone of etiolated cucumber hypocotyls and purified according to McQueen-Mason et al. (1992) with partial modifications using a cation exchange column combined with a hydroxyapatite column. The active fraction revealed a single silver-stained band with a molecular mass of 25 kDa by SDS–PAGE

Table 1 Effects of boiling, urea and expansin on the wall mechanical parameters of the frozen and thawed segments of cucumber hypocotyls

Samples	Conditions	ϕ (% h^{-1} gf^{-1})	y (gf)
Native	Control	1.29 ± 0.23^a	15.8 ± 0.5^b
Native	2 M urea	1.21 ± 0.22^a	14.3 ± 1.2^b
Native	8 M urea	0.28 ± 0.13^c	21.6 ± 1.4^d
Boiled	Control	0.38 ± 0.15^c	22.1 ± 0.9^d
Boiled	2 M urea	0.18 ± 0.09	22.5 ± 1.9
Boiled	10 μg ml^{-1} expansin	0.74 ± 0.18	14.7 ± 0.8
Boiled	20 μg ml^{-1} expansin	1.14 ± 0.15^a	15.0 ± 0.6^b

Yielding properties of cucumber walls were measured in buffer at pH 4.5.
Wall extensibility (ϕ) and yield tension (y) were calculated from each stress-extension rate curve.
The values are the means ± SD ($n = 5$).
^{a,b,c,d}The same letters are not statistically different from each other ($P > 0.05$).

analysis (Fig. 5A). The N-terminal amino acid sequence of this purified protein was determined to be D-Y-G-G-(C or W)-Q-S-(G)-H-A-. We concluded that this protein was a cucumber α-expansin (CsEXP1; Shcherban et al. 1995), although the fifth and the eighth amino acids were ambiguous.

The expansin thus purified restored the acid-facilitated creep of boiled walls to a similar extent (Fig. 5B) as reported previously (McQueen-Mason et al. 1992, McQueen-Mason and Cosgrove 1994). The SER analysis on the heat-inactivated walls reconstituted with expansin at pH 4.5 clearly demonstrated that the α-expansin could restore both the acid-induced increase in ϕ and the downward shift of y in the heat-inactivated FTPs. For full restoration, 20 μg ml^{-1} of expansin was necessary. It was apparent that the α-expansin participated not only in the pH-dependent regulation of ϕ but also in the acid-induced y shift (Fig. 5C, Table 1).

Effects of moderate concentrations (2 M) of urea on the SER characteristics

Moderate concentrations (2 M) of urea were proposed to act synergistically with expansin in acid-facilitated wall creep by weakening the hydrogen bonding between wall polymers in the cell wall. Thus the effects of urea on the SER properties were studied. Urea (2 M) enhanced the creep of native isolated walls as reported by McQueen-Mason and Cosgrove (1994). The process of the enhancement consisted of two major phases: the initial transient burst of the creep and the enhanced creep lasting >1.5 h. Fig. 6A represents the time courses of the urea-induced

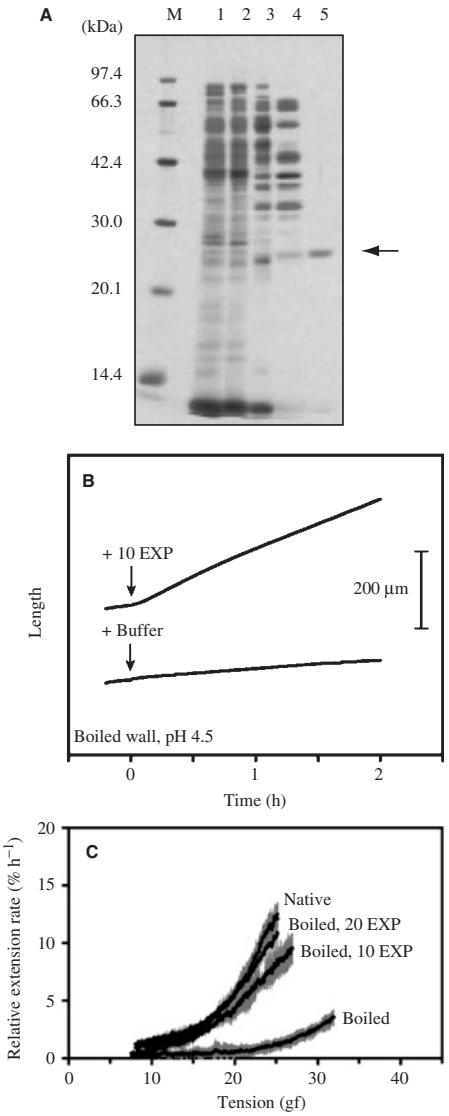

Fig. 5 Purification of expansin from cucumber hypocotyls, and extension of boiled and reconstituted cucumber walls. (A) Silver-stained SDS–polyacrylamide gel of active fractions from every purification step. Lane 1, 1 M NaCl extracts (1 μg); lane 2, ammonium sulfate precipitates (1 μg); lane 3, active fraction from the first Toyopearl SP column (1 μg); lane 4, active hydroxyapatite fraction; lane 5, active fraction from the second Toyopearl SP column (0.05 μg). The molecular masses of the standards are indicated on the left (M). The arrow indicates the active purified protein. (B) Boiled cucumber FTPs were clamped at a tension of 20 gf at pH 4.5 for 2 h and thereafter the bathing solution was replaced by the same buffer in the absence ('Buffer') or the presence of 10 μg ml^{-1} cucumber α-expansin (10 EXP) at the time indicated by the arrows. The bar represents 200 μm. (C) Effect of expansin on the SER curve. The SER curves of native and boiled FTPs were measured at pH 4.5. The boiled FTPs were reconstituted by adding expansin to the bathing solution at 10 (10 EXP) or 20 μg ml^{-1} (20 EXP). Each SER curve was drawn as the averaged curve from the data of five independent experiments. The gray zone behind each curve indicates the SD.

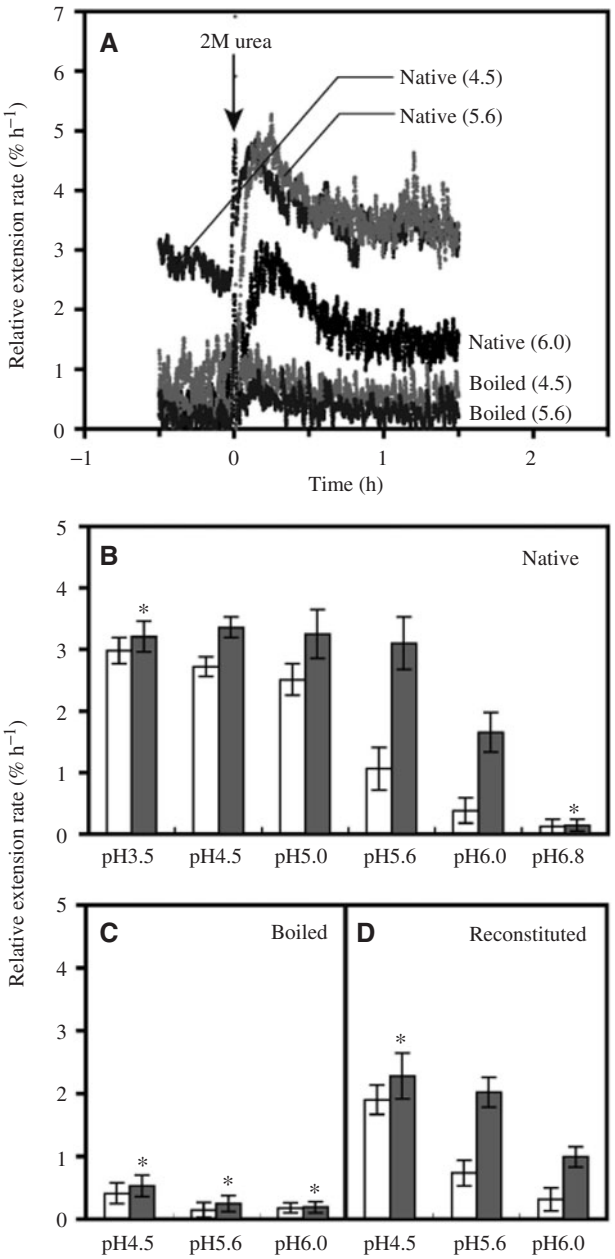

Fig. 6 Effects of 2 M urea on the creep rate of cucumber cell walls. (A) Native or boiled FTPs were extended under a tension of 20 gf in buffer at pH 4.5, 5.6 and 6.0 for 2 h; thereafter the bathing solutions were replaced by the same buffer solutions containing 2 M urea at the time indicated by the arrow. The pH dependence of the creep of native (B), boiled (C) and reconstituted FTPs (D) in the absence (open bars) and presence (filled bars) of 2 M urea. The creep tension was kept at 20 gf throughout. Boiled FTPs were reconstituted by adding 20 $\mu g\,ml^{-1}$ α-expansin to the bathing solution of boiled FTPs before creep measurements. Data are means for five independent experiments and the bars indicate the SD. The asterisk (*) indicates no statistical difference ($P > 0.05$) between the absence and presence of 2 M urea.

enhancement of the creep under a tension of 20 gf in different conditions. However, the rate of the long-lasting enhanced creep distinctly decreased with pH increase from 6.0 to 6.8, although there were no differences in the rate between pH 3.5 and 5.6. It showed a clear pH dependence of the urea-enhanced creep (Fig. 6B). The urea-induced increase in the rate of creep was maximal at pH 5.6 and was minimal at pH 3.5 and 6.8.

The creep of boiled walls was enhanced little by 2 M urea irrespective of pH (Fig. 6C). Both the initial transient and the long-lasting enhancement of creep were inhibited by heat treatments (Fig. 6A). In the heat-treated walls reconstituted with α-expansin, however, the creep was enhanced by 2 M urea pH dependently (Fig. 6D).

The treatment with 2 M urea caused distinct effects on the SER characteristics at pH 5.6 and 6.0, with only a small effect at pH 4.5 (Fig. 7). The effects of 2 M urea on the wall-yielding properties appeared to be equivalent to those of a 0.5 lower pH. Urea at 2 M apparently induced a downward y shift as well as an increase in ϕ at pH 6.0 (Fig. 7B, C).

Discussion

The SER characteristics that can be approximated by the Lockhart equation (Equation 1) are essential for the analysis of the yielding properties of isolated cell walls. However, the wall extensibility is indistinguishable from the yield tension by usual creep analysis (Cosgrove 1989, Tanimoto et al. 2000). These parameters were determined by the extensiometry of stepwise loading (Okamoto and Okamoto 1994, Taguchi et al. 1999, Ezaki et al. 2005). Manual stepwise extensiometry using weight loads is a useful and convenient method for SER analysis. However, the smallest step in the measurements was practically about 1.0 g and it took >30 min for extension to reach a stable state. Therefore, we could measure only a limited number of the rates of stable extension by this method. Under an acidic pH, only two to three points were available for estimation of a regression line to the high-rate extension of cucumber cell walls having a relatively low rupture tension. The rupture tension of cucumber walls was about 25 gf at pH 4.5, while it was 120 and 70 gf at pH 4.0 in cowpea and soybean, respectively (Okamoto and Okamoto 1994, Ezaki et al. 2005). Mechanical disturbance at weight loading seemed to interfere with the measurements. Fragile walls such as heat-denatured ones were often seriously damaged during measurements. The stress–strain analysis using the PCM could overcome these difficulties although ϕ is always higher than that estimated by manual extensiometry. The loading step in the PCM is not as steep as that in manual extensiometry using weight loads since the loading in the PCM is adjusted by a geared stepping motor. The maximum $d\tau/dt$ at the loading of 5 g was

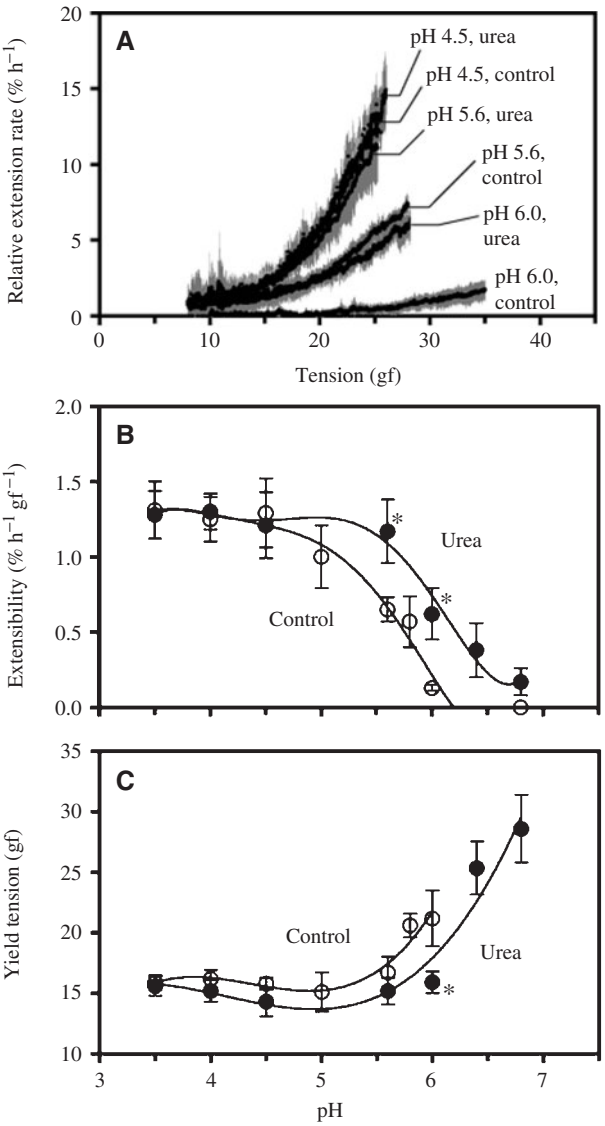

Fig. 7 Effects of 2 M urea on the SER characteristics of cucumber cell walls. (A) The SER curves of native FTPs were measured in the presence ('Urea') and absence ('Control') of 2 M urea under the indicated pH. The pH dependence of the wall extensibility and the yield tension are represented in (B) and (C), respectively. The asterisk (*) indicates a statistical difference ($P<0.05$) between the absence and presence of 2 M urea at the same pH.

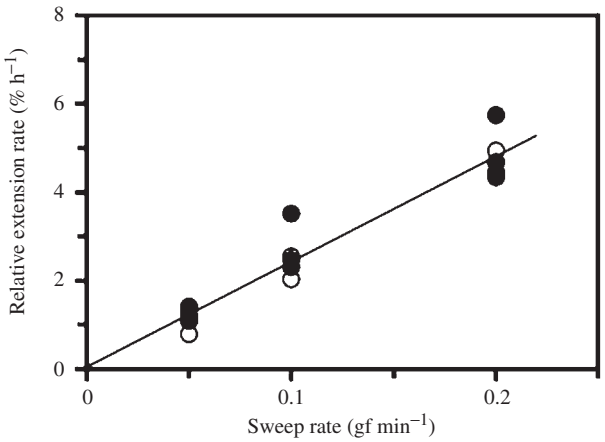

Fig. 8 Effects of the rate of ramped sweep on the values of the y intercepts ('h' in Fig. 2B) of the SER curves. The values of 'h' were calculated as the y intercepts of the regression lines to the low-rate extension (see details in the text). Filled circles and open circles indicate the values at pH 4.5 and pH 6.8, respectively. Three independent experiments were carried out under each condition.

approximately $0.5\,\mathrm{gf\,s^{-1}}$ using the PCM and $21\,\mathrm{gf\,s^{-1}}$ by manual extensiometry. The weight loading may evoke some opposing reactions within cell walls against an abrupt change in tension. A small perturbation in keeping a tension by a stepping motor might make cell walls easier to extend.

The ramped sweep method is preferable for measuring the SER curves with high resolution. However, the SER curves measured by the ramped sweep method always had an initial transient rise and constant rise proportional to the sweep rate (Fig. 8). These difficulties could be corrected adequately by processing using the numerical output of PowerLab. Both phenomena are considered to originate from the elastic properties of isolated cell walls because the wall extension can be approximated in general by the following equation (Lockhart 1965, Ortega 1985),

$$V = (1/l)(\mathrm{d}l/\mathrm{d}t) = \phi(\tau - y) + (1/\varepsilon)(\mathrm{d}\tau/\mathrm{d}t) \qquad (2)$$

where ε is the elastic modulus of cell walls. The equation indicates that changes in tension elicit the elastic responses of cell walls (the second term of the right side of Equation 2). The initial transient rise in v must be caused by abrupt changes in $\mathrm{d}\tau/\mathrm{d}t$ at the beginning of a ramped sweep, and the ensuing ramp ($\mathrm{d}\tau/\mathrm{d}t = \text{constant}$) gives a constant bias in the SER curve by $(1/\varepsilon)(\mathrm{d}\tau/\mathrm{d}t)$ (h in Fig. 2B). The dependence of the y-intercepts on the sweep rate at pH 4.5 is shown in triplicate (Fig. 8). The apparent elastic modulus estimated from the slope of the regression line was $0.40\%\,\mathrm{gf^{-1}}$ and did not differ significantly from the value at pH 6.8. It may be concluded that the corrected SER curves measured at a constant sweep rate within 0.05–$0.1\,\mathrm{gf\,min^{-1}}$ are equivalent to the curves measured by the stepwise method using the PCM and give more accurate information on the rheological properties of isolated walls (Fig. 2C).

The corrected SER curves of cucumber cell walls are roughly approximated with two regression lines. The wall extensibility ϕ and the yield tension y are estimated as

the slopes and the x-intercepts of the regression line to the high-rate extension (L2 in Fig. 2C), respectively (Ezaki et al. 2005). It is apparent that the acid-facilitated creep originated from the pH-sensitive properties of this high-rate extension. The present studies demonstrate that a downward shift of y as well as an increase in ϕ play important roles in the regulation of the acid-facilitated wall extension in cucumber FTPs. These characteristics are essentially the same as those reported in cowpea (Okamoto and Okamoto 1994) and in soybean (Ezaki et al. 2005). However, the low-rate extension undoubtedly takes parts in wall extension although the Lockhart equation does not define this extension (the extension under $\tau < y$).

The wall extension relevant to stem elongation is creep, i.e. the extension of cell walls under constant stress (Cosgrove and Cleland 1983, Pritchard et al. 1987, Rich and Tomos 1988, Nakahori et al. 1991, Maruyama and Boyer 1994). Fig. 3C indicates that the acid-facilitated creep is turned on by a pH decrease from 6.0 to 5.8 and increases markedly with further acidification if the applied tension is larger than 20 gf. In particular, a pH-linear change in the creep rate between 5.6 and 4.5 under the tension between 20 and 23 gf seems to be the important yielding characteristics of cucumber cell walls for the regulation of wall extension. The pH of native plant walls was estimated to be around 6.0 (Böttger et al. 1980, Mizuno and Katou 1991). Therefore, the changes in wall pH between 6.0 and 4.5 are thought to be necessary and suitable for the in vivo regulation of wall extension in cucumber hypocotyls. Under a tension <15 gf, the cell walls can be regarded to be in a state of the low-rate extension where the applied tension is always less than y irrespective of wall pH (Fig. 3B).

Boiling and/or high concentrations of urea fully suppressed both the acid-induced ϕ increase and downward y shift (Fig. 4, Table 1) that resulted in the inhibition of the acid-facilitated wall creep. These findings suggest the indispensable roles of wall proteins in the pH-dependent regulation of the wall-yielding properties and therefore the acid-induced promotion of wall creep. Expansin has been reported, by simple creep assays, to promote wall creep in an acidic condition (McQueen-Mason et al. 1992). It may be pertinent to consider that expansin should increase the wall extensibility pH dependently. However, these parameters of isolated cell walls could be estimated by the stress–strain analysis measuring the SER properties (Okamoto and Okamoto 1994) but not by the simple creep analysis (Cosgrove 1989). Thus, whether expansin modulates the wall extensibility ϕ or the yield tension y remains unknown. It was reported in cowpea hypocoytls that two kinds of wall proteins named yieldin and the ϕ-protein independently regulated y and ϕ, respectively (Okamoto and Okamoto 1995, Okamoto-Nakazato et al. 2000a). Thus yieldins or similar proteins were also presumed to participate in the regulation of the acid-induced wall extension in cucumber. Therefore, we did not expect that expansin fully restored not only the ϕ increase but also the y shift by reconstitution (Fig. 5C) even though no wall proteins from cucumber hypocotyls cross-reacted with anti-yieldin antibodies (unpublished). Cucumber α-expansin is capable of regulating the two different wall-yielding parameters of ϕ and y. These two parameters, however, might have different sensitivities to expansin because $>20\,\mu g\,ml^{-1}$ of expansin is necessary for full restoration of ϕ at pH 4.5 while $10\,\mu g\,ml^{-1}$ is sufficient for restoration of y (Table 1). The mode of action of expansin in ϕ regulation might be different from that in y regulation.

Expansin has been proposed to promote wall creep by disrupting non-covalent bonding (e.g. hydrogen bonding) within cellulose/matrix glucan polymers (McQueen-Mason and Cosgrove 1994, Cosgrove 2000, Darley et al. 2001). One line of experimental evidence was the finding that expansin-mediated wall extension was increased by 2 M urea because it could weaken hydrogen bonding between wall polymers (McQueen-Mason and Cosgrove 1994). However, in contrast to these observations, only a small promotion of wall creep occurred under acidic pH of <5.0 (Fig. 6B) and almost no difference was found in the SER curves between native and urea-treated walls at pH 4.5 (Fig. 7A). The high pH dependence of the enhancement of wall creep by 2 M urea and the low level of enhancement in boiled walls (Fig. 6A, C) suggest that 2 M urea does not directly weaken hydrogen bonding between wall polymers. If the disruption of hydrogen bonding in wall polymers were the primary cause of wall extension, the enhancement of wall creep would always be induced by treatment with urea. However, this is not the case. In the boiled walls reconstituted with expansin, the pH-dependent enhancement of wall creep by 2 M urea was recovered (Fig. 6D). Therefore, moderate concentrations (2 M) of urea are able to activate expansin possibly by causing structural modifications of the catalytic domain and/or wall polymer-binding domain of expansin. The inhibitory effects of 8 M urea on the SER characteristics are very probably due to denaturation of expansin as discussed by McQueen-Mason and Cosgrove (1994). Therefore which bonding is cleaved by expansin remains unknown. The nature of the yielding properties of cell walls requires further study.

Materials and Methods

Plant material

Seeds of cucumber (*Cucumis sativus* L. cv. Aonagachibai, Asashi Noen, Sobue, Aichi, Japan) were soaked in a solution

of 0.5 mM $CaSO_4$ for 8 h at 28°C, sown on moist sand and grown in the dark at 28°C for 3 d. Seedlings with hypocotyls 80 ± 5 mm in length were harvested and stored at –80°C until use.

Cell wall preparation

Specimens of the cell wall for extensiometry were prepared by essentially the same procedure as reported by Cosgrove (1989). Briefly, the frozen seedlings were thawed at room temperature and their hypocotyls were abraded with silicon carbite (320 mesh, Showa Chemicals Co. Ltd, Tokyo, Japan). Hypocotyl segments 15 mm long were excised with a razor from the elongation zone 5 mm beneath the cotyledonary node and pressed with a weight of 40 g for 5 min (FTPs). The heat treatment was carried out by dipping frozen–thawed segments in boiling water for 15 s before pressing them. This treatment was sufficient to inactivate expansin (McQueen-Mason et al. 1992), and was more drastic than the treatment (90°C for 15 s in 50% glycerol solution) to inactivate both yieldin and the putative ϕ protein in cowpea GHCs (Okamoto and Okamoto 1995).

Extraction and purification of expansins

Wall proteins were extracted and purified according to McQueen-Mason et al. (1992) with partial modification. Apical 30 mm cucumber hypocotyls were harvested and homogenized with a blender in 2 vols (w/v) of ice-cold 20 mM Na-acetate buffer (pH 4.5) containing 2 mM EDTA and 1 mM dithiothreitol (DTT). The homogenates were filtered through a nylon mesh (70 μm), and the filtrated residues were washed five times with the same buffer. The washed residues were suspended in HEPES buffer (pH 6.8) containing 1 M NaCl together with 2 mM EDTA and 1 mM DTT, and the suspension was stirred overnight at 4°C. Extracted proteins obtained by removing cell wall residues with filtration and centrifugation (20,000×*g* for 15 min) were precipitated with ammonium sulfate (20–60% saturated). The precipitated proteins were resuspended in 20 mM sodium phosphate (pH 6.8) and centrifuged at 20,000×*g* for 15 min to remove the insoluble proteins. The soluble proteins thus obtained were loaded onto a cation exchange column (Toyopearl SP 650S, Toso, Tokyo, Japan). The bound proteins were eluted with a linear gradient of NaCl from 0 to 250 mM. The active fractions were collected and loaded on to an hydroxyapatite column (CHI type I 20 μm, Nippon Bio-Rad Laboratories, Tokyo, Japan). The adsorbed proteins were eluted with a linear gradient of sodium phosphate from 20 to 300 mM. The active fractions were diluted in 5 vols of distilled water and loaded again onto a cation exhange column (Toyopearl SP 650S, Toso, Tokyo, Japan). The proteins were eluted with a linear gradient of NaCl after washing the column with 20 mM dimethylglutaric acid (pH 5.0). SDS–PAGE using a 15% acrylamide gel was carried out for every active fraction, and gels were silver stained. For all fractions except the second Toyopearl fraction, 1 μg of proteins were loaded on the gel. For the second Toyopearl fraction, the active fraction including 0.05 μg of proteins was concentrated with 10% trichloroacetic acid treatment and subjected to SDS–PAGE analysis. Expansin activity was assayed by the method of creep assay using boiled FTPs under a constant tension of 20 gf as reported by McQueen-Mason et al. (1992). Protein concentrations were estimated with Coomassie protein assay reagent (Protein Assay kit I, Nippon Bio-Rad Laboratories, Tokyo, Japan) using bovine γ-globulin as a standard. The N-terminal amino acid sequence of the purified proteins was determined as described previously (Okamoto-Nakazato et al. 2000b).

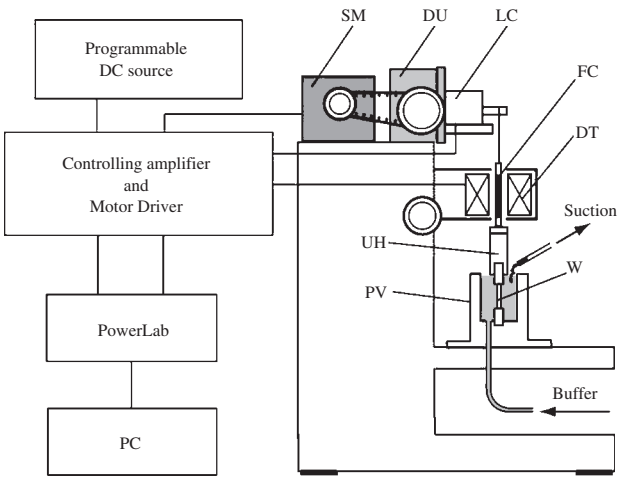

Fig. 9 Schematic illustration of the PCM. The wall specimen is pulled with the upward movement of the upper holder driven by a geared stepping motor. The motor movement is regulated to follow the commanded tension with a mechatronic system. The applied tension and extension of the wall specimen are measured with a load cell and a differential transformer, respectively. Both signals are recorded and calculated with PowerLab system, SM, stepping motor; DU, focusing drive unit of a metal microscope; LC, load cell; FC, ferrite core; DT, differential transformer; UH, upper holder; PV, perfusion vessel; W, wall specimen. See details in Materials and Methods.

Programmable creep meter

The automated extensiometer as shown in Fig. 9 was developed. The working principle of this equipment is not different from previous equipment such as the Instron (Cleland 1984) or Rheonocreepmeter (Tanimoto et al. 2000), i.e. a wall specimen is extended with a stepping motor-driven actuator and the applied tension is measured with a load cell. Our equipment, however, was developed from a manual extensiometer (Okamoto and Okamoto 1994). It is suitable for microstress–strain analysis of a cell wall specimen perfused with the desired dipping solutions. The system is designed to work as a tension clamp system by a mechatronic negative feedback. Commanded creep analysis can easily be carried out. Tension and material length can be also acquired as electrical signals that make it easer to be processed and analyzed with a PC system.

A wall specimen fixed to two pieces of vinyl chloride with cyanoacrylate glue (Aron-alpha, Konishi, Osaka, Japan) at both cut ends was clamped to polycarbonate holders. The upper holder with a ferrite core was hung on the sensing lever of a load cell (LVS-100GA, Kyowa Electronic Instruments, Chofu, Japan) and the lower holder was fixed in a perfusion vessel mounted on the stage. The tensile stress was imposed on the wall specimen by elevating the focusing drive unit of a metal microscope (BHMJ, Olympus, Tokyo, Japan) with a geared stepping motor (RK543AA-HA, Oriental Motor Co, Tokyo, Japan). The load cell was mounted on the focusing unit. The stepping motor was controlled in response to the difference between the calibrated output of the load cell and a commanded voltage. The length of the wall specimen was measured with a differential transformer (DTD-3, Seiyu Electronics, Kawasaki, Japan). The applied tension and the wall length were recorded every 2 s

with the PowerLab system (PowerLab 2/20, AD Instruments Japan, Nagoya, Japan) with a PC, and the rate of the wall extension was simultaneously calculated and recorded.

Stress extension rate (SER) analysis

FTPs were used for SER analysis using the PCM. The length of the segment undergoing extension (about 2.5 mm) was measured just before analysis. The wall specimens were initially bathed in 50 mM HEPES-NaOH pH 6.8 and subjected to a tension of 10 gf for 15 min. After release of the tension, the bathing solution was exchanged with a test solution, and then the tension for the SER analysis was applied.

Stepwise analysis. The tensional stress was increased stepwise from 2 gf up to the rupture tension. This procedure is essentially the same as that reported previously using a manual extensiometer (Okamoto and Okamoto 1994, Okamoto-Nakazato et al 2000b, Ezaki et al. 2005). The increment and duration of each step were programmed to be 2 gf and 30 min, respectively. The step duration of 30 min was almost sufficient for the wall creep to reach steady state after the initial transient burst of the extension rate.

Ramped sweep analysis. A linear ramped tension was applied to the wall specimen up to rupture. The minimum step of commanded signal generated with a programmable DC source (7651, Yokogawa Electric Works, Tokyo, Japan) in ramp-on mode was equivalent to 10 mg. The SER curve could be measured simultaneously with a single ramped sweep.

Acknowledgments

We thank Mr. Syuzo Ishikawa and Mr. Tatsuharu Torii at the Technical Center of Nagoya University for their excellent technical work (electronic engineering by S.K. and mechanical engineering by T.T.) in developing a programmable creep meter. In collaboration with them, K.K. designed the PCM. This work was founded in part by Daiko Foundation (N.K.). K.K. would like to dedicate this paper to the memory of the late Professor Dr. Achim Hager who often encouraged him.

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(Received August 4, 2006; Accepted September 26, 2006)