Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(7):1460-1466

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Study on the cross-links between wall polysaccharides by dehydrodimers of hydroxycinnamic acids during the cessation of cell elongation

Jiang-Gang Yang, Ying-Ying Ma, Yan Lin, Qiu Lin and Hechuan Wu

Department of Bioengineering, College of Bioengineering, Sichuan University of Science and Engineering, Zigong, China

---

ABSTRACT

In this paper, the position of crossing linkage between cell wall polysaccharides was studied by determining the digestibility of pectin-depleted walls, the contents of hydrocinamic acids and their dimmers in each fraction of elongated and non-elongated cells. The results showed that Ox. sol. fr. was composed mainly of pectin polysaccharides while 4K sol. fr. was composed both of pectin and hemicellulosic polysaccharides. The evidence that higher percentage of acid sugar and higher concentrations of hydrocinnamic acids were present in 4K sol. fr. of non-elongtated cell walls suggests that more pectic polysaccharides are cross-linked with other polymers in non-elongated walls. A higher digestibility of elongated cell-walls suggests that xyloglucan in the walls are easy to creep by cellulase or expansin while the non-elongated cell-walls show a cellulase-resistant property due to the cross-links between polysaccharides such as pectin and xyloglucan. The facts suggest that ferulic acids are not only ester-linked to pectic polysaccharides but also hemicellulose polysaccharides. These esterified hydroxycinamic acids may form complex cross-links between pectins, xyloglucans, or pectin and xyloglucan, therefore, involving in the cessation of cell elongation of suspension-cultured cells of Mentha.

Keywords: Cell wall, fractionation, elongation, cessation, cross-link, polysaccharide, hydrocinnamic acid

---

INTRODUCTION

The growing plant cell wall has unique physical property that permit it to elongate by a mechanism of slow polymer flow (creep) which cause wall loosening. This elongation by a mechanism had long been hypothesized as the break down of the bond between xyloglucan (or arabinoxylan) and cellulose catalyzed by expansin [1] or as the polysaccharides-modifying by wall loosening enzymes such as endo-β-glucanase [2-3]. XET [4]; cellulase [5]. On the other hand, the growing plant cell wall contains polymers which bear a small property of phenolic side-chains. These side-chains appear to be subject *in vivo* to oxidative phenolics coupling and thus to participate in cross-linking reactions that may be high significant in the control of wall extensibility (and therefore in the cell growth) and of enzymic digestibility [6-7]. The mechanism of the cessation of cell elongation was hypothesized as the formation of the dehydroxycinnamic acids between wall polysaccharides catalyzed by wall-bound peroxidase [6, 8-9].

In the view of this possible function of cell-wall phenolics in the cessation of cell elongation, biochemical interesting is focused on: where they are linked within cell-wall, how they are cross-linked with cell-wall polysaccharides, and what kind of phenolics are involved in cell-wall. It has been reported that the occurrence of the hydroxycinnamic acids such as ferulic acid (FA) and ρ-coumaric acid (PCA) are ester-linked to arabinoxylans in grasses [10], to pectic polysaccharides in spinach [11-12], in sugar beet]13-14] and to xyloglucans in bamboo [15]. We have presented the evidence that the formation of dehydrodimers of hydrocinnamic acids is a peroxidase-catalyzed process in cessation of the cell elongation in *Metha* suspension culture [8-9, 16]. In this paper, the walls of non-elongated and elongated cells of suspension cultured *Mentha* were fractionated by oxalate buffer, 4% KOH, 24% KOH, and the contents of acidic sugar, neutral sugar, total sugar in each fraction were determined.

The constituent of monosaccharides of matrix polysaccharides in each fraction was also compared between both two kinds of cell-walls. Furthermore, amounts of wall-bound ferulic acid, caffeic acid, and 5, 5-dehydrodiferulic acid (DFA) in pectin, pectin-depleted walls, and cellulase insoluble fraction of pectin-depleted walls of non-elongated cells were investigated. The enzyme digestibility of both pectin-depleted of non-elongated and elongated cell walls by cellulase was also compared.

EXPERIMENTAL SECTION

Cell suspension culture of *Mentha*

Cell suspension cultures were maintained as described in before (Yang *et al.*, 1999). Non-elongated cell growth was initialed by transferring 5 ml of 10-12 days cultured suspensions which was maintained in medium containing 2000 μg/L to the medium containing low concentration of 2,4-D (2μg/L). Elongated cells were cultured in medium containing 2000 μg/L of 2, 4-D. Both elongated and non-elongated cell were harvested at day 10-12 for cell-walls preparation. The preparation and purification of cell walls were carried out as shown as previously [8-9].

Fractionation of purified cell walls

The purified cell-walls from non-elongated and elongated cells were fractionated as show in Fig.1. The purified cell-walls from elongated and non-elongated cells were extracted six times with 40 mM ammonium oxalate buffer (pH2.0) at 70 ℃ for 1 h each time until the polysaccharides was not detected in the extracts by the phenol-H_2SO_4 method. The combined supernatant was concentrated by vacuum evaporation to one-third of the original volume, then dialyzed against with water at 4 ℃ for 24 h. Finally, it was lyophilized to obtain the ox. sol. fr. as show as in Fig.1. The buffer insoluble fraction (pectin-depleted cell-walls) was further extracted with 4% KOH at room temperature for 24 h, the pH of the extracts was adjusted to 6.0 with HCI, dialyzed and lyophilized to obtain 4K sol. fr. The residues of the 4% KOH was finally extracted with 24% KOH at room temperature for 24 h, the pH of extracts was adjusted to 6.0 with HCI, then dialyzed and lyophilized to obtain 24K sol.fr. [17].

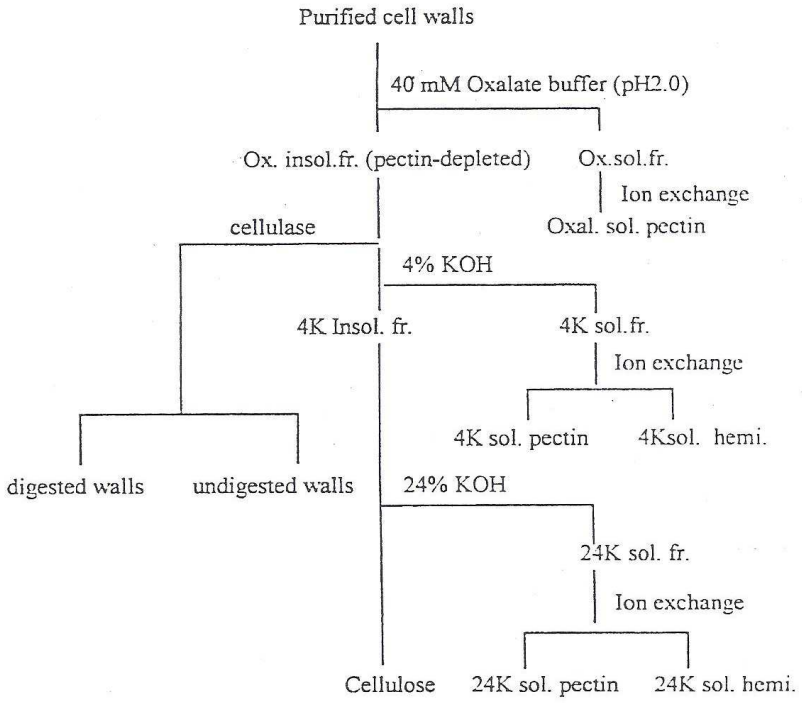

Fig 1. Fractionation of the purified cell walls by oxalate buffer, 4% KOH, 24%KOH and hydrolysis of the pectin-depleted walls (Ox. insol. fr.) by cellulase

Determination of acidic sugar, neutral sugar, and total sugar content in Ox. sol. fr., 4K sol. fr., and 24K sol. fr.: 100 mg of each fraction was respectively resolved in 100 ml distilled water, the sugar content in the solution was determined respectively. Acidic sugar was carried out by the *m*-hydroxydiphenyl method [18] as follows. The solution (0.5 ml) were added to a test tube (Φ16 mm) and cold in ice water. Concentrated H_2SO_4 solution (3 ml) which contained sodium tetraborate decahydrate were added to test tube followed by stirring, then putted it in boiling water for 5 min and stooped the reaction by decreasing the temperature immediately with ice cold water. After laying for 20 min in room temperature, the absorbance at 520 nm was detected by using α-D-galacturonic acid as a standard. Neutral sugar content was measured by the orcinol method reported by Winzler [19]. The solution (0.5

ml) was added to a test tube (Φ16 mm) and cold in ice water. Then, 4.5 ml mixed solution of 60% H_2SO_4 with 1.6% orcinol cooled solution (7.5:1) was added to test tube followed by stirring, then putted it in to block heater at 80 ℃ for 15 min and stopped the reaction by decreasing the temperature immediately with ice cold water. After laying for 20 min in room temperature, the absorbance at 505 nm was detected by using D-(-)-arabinose as a standard. Total sugar content determinations were finished by phenol- H_2SO_4 method (Hodege and Hofreiter, 1962) as follows. The solution (0.5 ml) was mixed with 1 ml 5% (w/v) phenol solution in a test tube (Φ, 16 mm) by stirring. After laying for 30min in room temperature, the absorbance at 490 nm was detected by using glucose as a standard.

Ion exchange chromatograph of each fraction

To determine the constituent sugar, each 100 mg extracts of Ox. sol. fr. 4K sol. fr. and 24K sol. fr. were respectively purified by ion exchange chromatograph (DEAE-Toyopearl, Maruyama *et al*., 1999) as follows. Each 100 mg extracts were dissolved in 10 mM phosphate buffer solution (pH 6.0), the insoluble material removed by filtration. The filtrated solution was loaded onto a DEAE-Toyopearl column (2.8×20 cm) pre-equilibrated with 10 mM phosphate buffer (pH 7.0), and eluted at 100 ml hr^{-1} with same buffer (three times volumes of column bed) to obtain neutral sugar as shown in Fig.1 (hemicellulose fraction: 4K sol. hemi., 24K sol. hemi.) and then a linear gradient of 0 to 500 mM NaCl containing same buffer solution. Appropriate fractions were combined, dialyzed and lyophilized to obtain acidic sugar (pectin fraction: Ox sol. pectic, 4K sol. pectic, 24K sol. pectic).

Analysis of constituent sugar of each fraction

The analysis of constituent sugar of each fraction was carried out by the method of Nevins *et al.* [20] as follows. Putting 10 mg samples to test tubes, then 3 ml of 2N trifluoroacetic acid solution containing 0.1 mg methy-β-D-glucoside (internal standard) was added followed by filling with N_2. Then the tube were sealed and hydrolyzed at 121℃ for 1 h, then dried by evaporation. The dried sample was resolved in 0.2 ml water with 1 ml $NaBH_4$ solution (1g NaBH4/50ml DMSO), and kept for 90 min at 40℃. Finally, samples were acetylated by addition of 0.2 ml of 1-methylimidazol and 2 ml of acetic anhydride at room temperature for 10 min. The monosaccharides in the samples after they were converted to their respectively alditol acetates were measured by GLC.

Cellulase hydrolysis of the cell-walls

To estimate the digestibility of cell walls by cellulase (EC 3.2.1.4), 1 g of pectin-depleted cell-walls were resolved in 100 mM of 0.1% NaOH solution (pH 4.7) containing 0.02%$NaBH_4$, 0.1g cellulase, and then incubated for 24h. The reducing sugar was determined at different times by Smoggy method by using glucose as a standard.

Analysis of esterified hydroxycinnamic acids and their dehydrodimers in the cell-walls

The Methods of qualitative and quantitative analysis were carried out as described before [8-9, 16].

RESULTS AND DISCUSSION

Contents of acidic sugar, neutral sugar, and total sugar in the soluble fractions (Ox. sol. fr. 4K sol. fr. and 24K sol. fr.)

Table 1. Sugar content of each soluble fraction from the walls of elongated and non-elongated cells

Cell wall component(mg/gcell-walls)			sugar content（% of total sugar） acidic sugar		neutral sugar		total suagr*	
	N	E	N	E	N	E	N	E
Ox.sol.fr.	107±34	122±66	38.80 (88.34%)	33.92 (97.14%)	5.12 (11.66%)	1.00 (2.86%)	43.92 (40.18%)	34.92 (31.73%)
4K sol. fr.	226±94	226±59	14.50 (60.62%)	11.34 (56.40%)	8.42 (39.38%)	8.76 (43.60%)	22.92 (19.35%)	20.10 (20.24%)
24K sol. fr.	130±63	226±59	——	——	——	——	——	——

**, detected by phenol-H_2SO_4 method，–，not performed；E，elongated walls；N, non-elongated walls；data means ±SE（n=3）.*

The table 1 shows the polysaccharides amounts and composition fractionated by oxalate buffer and KOH solution from the walls of elongated and non-elongated cells. The amount of pectic polysaccharides (Ox. sol. fr.) in the walls of elongated and non-elongated cells was higher than that in the walls of non-elongated cells. The amount of 4K sol. fr. from the walls of non-elongated cells was same as that from elongated cells, while the amount of 24K sol. fr. from the walls of elongated cells was lower than that from non-elongated cells. However, there were no significant differences in the amount of cell wall components. The acidic sugar (or neutral sugar) in Ox. sol. fr. of

non-elongated walls was 88.34% (or 11.66%) of total sugar, while that in the Ox. sol. fr. of elongated cell-walls was 97.14% (or 2.86%) of total sugar.

The acidic sugar (or neutral sugar) in 4K sol. fr. of non-elongated cell-walls was 60.62% (or 39.38%) of total sugar while that in elongated cell-walls was 56.4% (or 43.60%) of total sugar. These results showed that Ox. sol. fr. was mainly composed pectic polysaccharides while 4K sol. fr. was composed both of pectic and hemicellulosic polysaccharides. On the other hand, the evidence that higher percentage of acid sugar was present in 4K sol. fr. of non-elongated cell-walls suggested that more pectic polysaccharides were cross-linked with other polymers in non-elongated walls.

Monosaccharides composition of matrix polysaccharide in walls of elongated and non-elongated cells

Table 2. Neutral sugar ratio of each fraction by DEAE-Toyopearl (n.d.，not detected；tr，trace）

	Neutral sugar (molar ratio, %)											
Cell walls fraction	Glc		Xyl		Gla		Rha		Ara		Man	
by DEAE-toyopearl	N	E	N	E	N	E	N	E	N	E	N	E
Ox. sol. pectin	3	tr	20	28	23	12	8	5	45	55	n.d.	n.d.
4K sol. pectin	8	6	23	35	15	11	8	5	46	43	1	tr
4K sol. hemi.	20	21	26	23	4	6	1	1	48	48	1	1
24K sol. pectin	15	12	25	27	18	18	6	6	36	36	tr	n.d.
24K sol. hemi.	39	39	43	46	2	2	tr	tr	14	16	1	tr

To determinate monosaccharides composition of matrix polysaccharide in each fraction from elongated and non-elongated cell-walls, it was further purified by ion exchange chromatography as shown in Fig.2. The ion exchange chromatography of Ox. sol. fr. of both elongated and non-elongated cell-wall gave only absorbed acidic polysaccharides (Ox. sol. pectin). The elution patterns of the ion exchange chromatography of Ox. sol. fr. of both kinds of walls was shown in Fig. 2A where one main peak was obtained between 0.18～0.20 and 0.21～0.25 mM NaCl, respectively. This result supported that the extract (Ox. sol. fr.) composed mainly pectic polysaccharides as detected as acidic sugar.

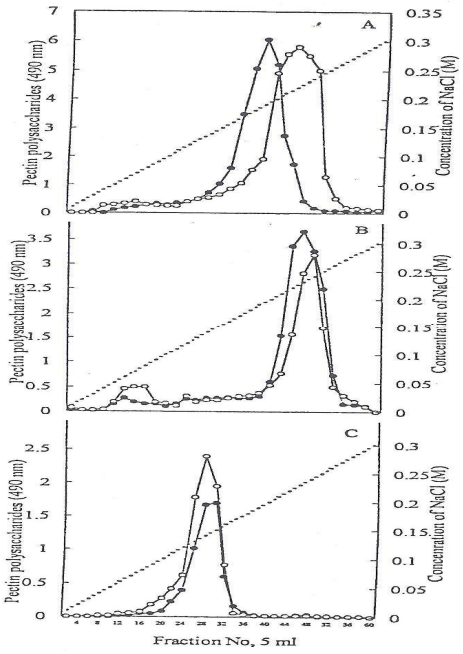

Fig 2. The ion exchange chromatography and elution pattern of acidic pectin substance in Ox. sol. fr.(A), 4K sol. fr.(B) and 24K sol. fr.(C) of elongated and non-elongated cells

The ion exchange chromatography of the extracts of 4K sol. fr. and 24K sol. fr. of both walls gave non-absorbed hemicellulosic (4K sol. hemi. and 24K hemi.) and absorbed pectic polysaccharides (4K sol. pectin and 24K sol. pectin). The elution pattern of acidic pectin substance in 4K sol. fr. and 24K sol. fr. of elongated and non-elongated

cells were shown in Fig.2B and 2C, respectively. One main peak from each extract of 4K sol. fr. and 24K sol. fr. of elongated and non-elongated cell-walls was obtained between0.22～0.25 and 0.12～0.16 mM NaCl, respectively. This result supported that the extracts (4K and 24K sol. fr. composed both pectic and hemicellulosic polysaccharides as detected as a mixture of neutral sugar and acidic sugar.

Monosaccharides composition of matrix polysaccharides in each purified fraction (Ox. sol. pectin, 4K sol. hemi. and 24K sol. hemi.) from both elongated and non-elongated cells were showed in Table 2. The molar ratio of xylose (23%) and arabilose (46%) in Ox. sol. pectin fr. of non-elongated walls were lower than that of elongated walls (28% and 55%, respectively) while the ratio of galactose was 11% higher than that of elongated walls. The ratio of xylose in 4K sol. pectin fraction of non-elongated walls (23%) was lower than that of elongated walls (35%), while the ratio of others in 4K sol. pectin and 24 K sol. pectin fractions between non-elongated and elongated cell-walls were same. The ratio of neutral sugar in 24K sol. hemi. fr. between elongated and non-elongated walls were also same. These results showed that there was no significant difference in composition of matrix polysaccharides and neutral monosaccharides (glucose, xylose, galactose, rhamnose, arabinose, and mannose) of the cell wall matrix polysaccharides between non-elongated and elongated cells as shown in Table 2.

It has been previously reported that elongated cells secreted arabinogalactans and acetylated xyloglucans as major pectic and hemicellulosic component of extracellular polysaccharides [21-22]. A further experiment identified the same acetylated xyloglucan in the cell wall polysaccharides of both elongated and non-elongated cells (unpublished data). Therefore, it is possible that the cell walls of *Mentha* (elongated and non-elongated cells) have essential wall extensibility, because they have an acetylated xyloglucan which causes considerable rheological changes in structure of xyloglucan in primary cell walls and promotes wall-loosening. The cells cultured in the low 2,4-D condition cannot elongate even if they have acetylated xyloglucan because the wall polysaccharides are cross-linked by phenolic acids through peroxidase-mediated oxidative coupling processes, and consequently, resulted in the cessation of cell elongation and wall rigidification.

The digestibility of pectin-depleted walls of elongated and non-elongated cells by cellulose

To determine the digestibility of cell-walls by cellulose, oxalate buffer insoluble walls (pectin-depleted walls) were used. The digestibility of cell-walls by cellulose between elongated and non-elongated cells was compared. As shown in Fig.3, the pectin-depleted walls of non-elongated cells (culture for 12 days) show a lower digestibility than that of elongated cells. The amount of reducing sugar hydrolyzed by cellulose from non-elongated cell-walls was only two-thirds that from elongated cell-walls showed a higher digestibility, suggesting xyloglucans in the walls may be easy to be hydrolyzed by cellulose [5], endo-glucanase [2], and XET [4], or/and to be crept by expansin [1] *in vivo* and therefore they can elongated by expansin or enzymes which cause wall-loosening. On the other hand, the non-elongated cell-walls showed a cellulose-resistant property similar to the driselase-resistant property in spinach cell-walls, suggesting that xyloglucans in the walls are tightly cross-linked by other polysaccharides such as pectin and xyloglucan probably through dehydrodimers of ferulic and caffeic acids. Therefore, the cells can not elongate [8-9].

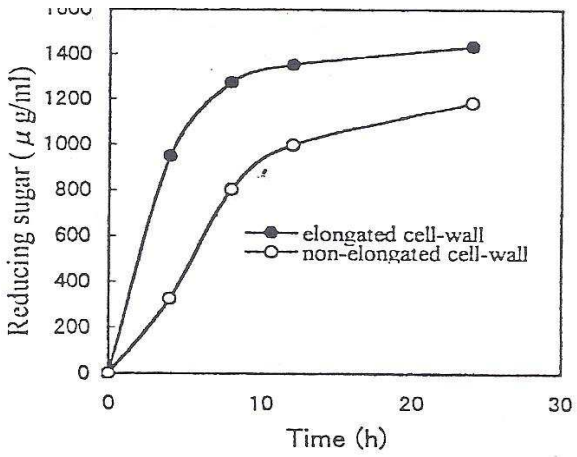

Fig 3. Time course for cell-wall digestion by cellulase

Table 3. Amount of ferulic acids, cafeic acids, and 5, 5-deferulic acids released from pectin, pectin-depleted walls, undigested walls of non-elongated cells

Fractions	FA (μg/g)	CA (μg/g)	5,5-DFA(μg/g)
pectin(Ox.sol.fr.)	11.3	50.5	n.d.
pectin-depleted walls (Ox. insol.fr.)	294.84	317.44	38.99
undigested walls (cellulase insol.fr.)	174.5	*	74.5

*n.d., not detected. *, not carried out.*

The caffeic acid was determined by the relative response factor of caffeic acid to ferulic acid (RRF=1.26) in the experimental condition. The amounts of ferulic acid, caffeic acid, and 5, 5-DFA in cellulase soluble fr. were not carried out.

We have reported that non-elongated cell-walls contain higher concentrations of phenolics and dehydrodimers of ferulic and caffeic acids [8-9, 16]. In the present study, the low digestibility of pectin-depleted cell-walls supported that higher concentration of cross-links between cell-wall polymers by phenolic substances present in non-elongated walls. This result in a decrease in enzymatic digestibility of cell-wall as has been proposed previously [5, 23].

Hydroxycinnamic acids and their dimmers in each fraction of non-elongated cell-walls

Caffeic acid and ferulic acid were found as main monomeric hydroxycinnamic acids in the pectin polysaccharides (Ox. sol. fr. as shown in Fig. 1). Pectin-depleted walls (oxalate buffer insoluble walls), and cellulase undigested fraction of pectin-depleted walls (oxalate buffer insoluble walls) of non-elongated cells. The dehydrodimers of ferulic and caffeic acids were also detected. The amount of FA and CA in ox. sol. fr. as shown in Table 4 was 11.30 or 50.50 μg/g pectin polysaccharides, respectively. These suggested that FA and CA are ester-linked to pectic polysaccharides as reported in spinach cell-walls of dicotyledons [11-12]. However, more hydroxycinnamic acids were found in pectin-depleted walls (Ox. insol. fr.) which gave a mixture of acidic and neutral sugar (pectic polysaccharides and xyloglucan) when it was hydrolyzed by 4% KOH as mentioned above. The amounts of FA, CA and DFA in pectin-depleted were 294.82, 317.44 or 38.99 μg/g of the pectin-depleted walls, respectively.

These results suggest that the possibility that FA and CA were not only esterified to pectin polysaccharides of spinach cell-walls of dicotyledons [11-12, but also esterified to xyloglucan as reported for bamboo cell-walls of monocotyledons [15] because if they esterified only to pectin saccharides, they must be extracted by oxalate buffer. In addition, the facts that 4% KOH hydrolysate of pectin-depleted walls (Ox. insol. walls) contained both acidic sugar, neutral sugar. FA, CA, and DFA were released from the pectin-depleted walls by 2 M NaOH suggest another possibility that formation of cross-links between pectic polysaccharides and xyloglucan through their esterified FA and CA because the 4% KOH solution usually release the hemicellulose polysaccharides such as xyloglucan and xylan from the cell-walls of dicotyledons plant [2]. To our knowledge, this is the first time that the evidence of hydroxycinnamic acids ester-linked to xyloglucan was reported in dicotyledons plant.

On the other hand, FA (174.50 μg/g) and DFA (74.50 μg/g) were also detected from the cellulase undigested fraction of oxalate buffer insoluble walls. This result supports the hypothesis that low cellulose-digestibility of pectin-depleted walls (Ox. insoluble fr.) of non-elongated cells is due to the complex cross links between wall polysaccharides probably xyloglucans or xyloglucan and pectic polysaccharides because cellulose cut only xyloglucan molecular and can not cut the cross-linkages [5]. Therefore, this result showed the formation of cross-links of wall bound phenolics results in the cellulose (dries-resistant) property of cell wall xyloglucan, therefore, involving in the cessation of cell elongation.

CONCLUSION

The results of component and neutral sugar composition of wall matrix polysaccharides between non-elongated and elongated cell showed that there was no significant difference. These results show that a change in the superstructure was involved. Ox. sol. fr. of the wall was composed mainly pectin polysaccharides, while the 4K sol. fr. of the walls was composed with both pectin and hemicellulosic polysaccharides. The evidence of higher concentration of hydroxycinnamic acids and higher percentage of acidic sugar in 4K sol.fr. of non-elongated wall suggests that more pectin polysaccharides were cross-linked with other polymers in the wall. A higher digestibility of elongated wall suggests xyloglucan in the walls were easy to creap by cellulose or expansin, while the non-elongated wall showed a cellulose-resistant property due to the cross-links between polysaccharides such as pectin and xyloglucan. The facts suggest that ferulic and cafeic acids were not only ester-linked to pectic polysaccharides but also to hemicellulosic

polysaccharides. These esterified hydroxycinnamic acids may form cross-links between pectins, xyloglucans, or pectin and xyloglucan. Therefore, feruloylation, caffeoylation and the dimerization of feruloylated and caffeoylated polysaccharides are involved in the cessation of cell elongation.

Acknowledgment
This paper was financially supported by the Fund of Sichuan University of Science and Engineering (13jzk07; 2011RC01; 2012RC14); Students' Innovation Fund of Sichuan Provincial Department of Education (201310622019); and the foundation of Sichuan Provincial Social Science Planning Office (SC13BJ12).

REFERENCES

[1]DJ Cosgrove, *Annu. Rev. Plant Physiol. Plant Mol. Bio.*, **1999**, 50(6), 391-417.
[2]T Hayashi, *Physiol. Plant Mol. Biol.*, **1989**, 40(1), 139-168.
[3]T Matumoto; F Sakai; T Hayashi, *Plant Physiol.*, **1997**, 114(2), 661-667.
[4]SC Fry; RC Smith; KF Renwick; DJ Martin; SK Hodge and KJ Matthews, *Biochem. J.*, **1992**, 282(3), 821-828.
[5]SC Fry, *Physiol. Plant.*, **1989**, 75(4), 532-536.
[6]SC Fry, *Annu. Rev. Plant Physiol.*, **1986**, 37(1), 59-64.
[7]SC Fry, The growing cell wall: Chemical and Metabolic Analysis, Longman and Wiley Publishers, London; New York, **1988**, 15-28.
[8]JG Yang; T Uchiyama, *Biosci. Biotechnol. Biochem.*, **2000a**, 64(8), 1572-1579.
[9] JG Yang; T Uchiyama, *Biosci. Biotechnol. Biochem.*, **2000b**, 64(4), 864-864.
[10]Y Kato; DJ Nevins, *Carbohydr. Res.*, **1985**, 147(1), 69-85.
[11]SC Fry, *Biochem. J.*, **1982,** 203(2), 493-504
[12]SC Fry, *Planta*, **1983**, 157(6), 111-123.
[13] A Bacic; PJ Harris; BA Stone, Academic Press. New York, **1998**, 297-371.
[14]TBT Lam; K Liyama; BA Stone, Primary and secondary walls of grasses and other forage plants: taxonomic and structural consideration. In Akin, DE Ljundahl; MG Wilson; PJ Harris, eds. Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier, New York, **1990**, 43-69.
[15]I Ishii; T Hiroi; *Carbohydr. Res.*, **1990**, 206 (2), 297-310.
[16]JG Yang; S Miyao; T Uchiyama, *Biosci. Biotechnol. Biochem.*, **1999**, 63(9), 1522-1527.
[17]S Kamisaka; S Takada, K Takahashi; K Shibata, K. *Physiol. Plant.*, **1990**, 78(1), 1-7.
[18]PK Kinter; JP Van Buren, *J. Food Science*, **1982**, 47(3), 756-759.
[19]R Winzler; R Sederoff; D Glick, In: Methods of biochemical analysis. eds. By Glick, D. Wiley InterScience. **1955**, 279-293.
[20]DJ Nevins; PD English; T Alberheim, *Plant Physiol.*, **1967**, 42(7), 900-906.
[21]K Maruyama; C Goto; M Numata; T Suzuki; Y Nakagawa; T Hosino; T Uchiyama, *Phytochem.*, **1996**, 41(5), 1309-1341.
[22]K Maruyama; H Yamamoto; T Uchiyama, *Biosci. Biotechnol. Biochem.*, **1998**, 62(11), 2223-2225.
[23]F Eraso; RD Hartley, *J. Sci. Food Agric.*, **1990**, 51(2), 163-170.