JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Methyl Esterification Divergently Affects the Degradability of Pectic Uronosyls in Nonlignified and Lignified Maize Cell Walls

JOHN H. GRABBER* AND RONALD D. HATFIELD

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1925 Linden Drive West, Madison, Wisconsin 53706

Nonlignified cell walls from *Zea mays* (L.) cell suspensions were incubated with and without pectin methylesterase (PME) and a portion were artificially lignified to assess how methyl esters influence the release of pectic uronosyls and total sugars from cell walls by fungal enzymes. Treatment with PME reduced uronosyl concentrations from 97 to 92 mg/g, reduced uronosyl methylation from 57% to 21%, and increased Klason lignin concentrations in artificially lignified cell walls from 99 to 116 mg/g. Although PME treatment slightly enhanced uronosyl release from nonlignified cell walls, it reduced uronosyl release from artificially lignified cell walls by 55% after 4 h and by 7% after 72 h of enzymatic hydrolysis. Pectin hydrolysis in PME treated cell walls was probably impaired by enhanced benzyl ester cross-linking of uronosyls to lignin via quinone methide intermediates. Variations in uronosyl methylation had little effect on the overall release of total sugars from cell walls.

KEYWORDS: ***Zea mays*; quinone methide intermediate; benzyl ester; cross-link; polygalacturonic acid; pectin; lignin; degradability; digestibility; pectinase; polygalacturonase**

INTRODUCTION

Galacturonic acid comprises 80% or more of the total uronosyls in primary cell walls and they are the major component of pectins in both dicots and grasses (*1*−*5*). Polygalacturonic acids deposited into cell walls are highly methyl esterified but most of these groups are subsequently removed by pectin methylesterase (PME) as tissues complete elongation and undergo lignification (*6*). Although galacturonans are among the most rapidly and extensively degraded of cell wall polymers (*5*, *7*), their enzymatic hydrolysis can be enhanced by demethylation via PME (*8*). Demethylation of galacturonans in lignifying tissues may, however, limit degradability because of enhanced formation of benzyl uronate cross-links. These cross-links are formed by the nucleophilic addition of uronosyl acid groups to quinone methide intermediates (**Figure 1**), which are formed by β-*O*-4 coupling of monolignol and lignin radicals during lignin polymerization (*9*). The addition of uronosyls to quinone methide intermediates is blocked by methyl esterification; hence demethylation by PME should enhance benzyl ester cross-linking and restrict pectin degradation as has been observed when xylans are oxidatively cross-linked to lignin by ferulate (*7*, *10*). Although benzyl ester cross-linking has been widely investigated with regard to lignin-carbohydrate complex formation and delignification of woody species (*9*), its influence on cell wall degradability is not known. In this study, the potential impact of benzyl-uronate cross-linking on pectin and cell wall hydrolysis was modeled by using PME to vary the methylation of uronosyls prior to artificial lignification of primary maize cell walls. In previous studies, this cell-wall model system was successfully used to explore how variations in ferulate cross-linking, lignin composition, and lignin polymerization conditions influence the enzymatic degradation of cell walls (*7*, *10*−*13*).

MATERIALS AND METHODS

Cells from suspension cultures of maize (*Zea mays* L. cv Black Mexican) were ruptured by a Parr Nitrogen Bomb and washed sequentially with PIPES buffer (10 mM; pH 7), aqueous $CaCl_2$ (100 mM), and water to remove cytoplasmic debris and loosely bound peroxidases (*14*). Wet cell walls (15 g; ca. 370 mg dry weight) were stirred in 30 mL of 100 mM NaCl-50 mM PIPES buffer (pH 7.5) with and without PME (1000 units; Sigma P5400) for 5 h. After incubation, cell walls were pelleted by centrifugation (2500 *g* for 10 min) and then washed by suspension in water (30 mL) before pelleting by centrifugation. The wash cycle was repeated three times and then cell walls were transferred to 125 mL Erlenmeyer flasks. Cell walls were then stirred in 30 mL of HOMOPIPES buffer (25 mM; pH 4.0 with 3 mM $CaCl_2$) and artificially lignified by adding (2 mL/h) separate solutions of coniferyl alcohol (0.42 mmol) and H_2O_2 (0.55 mmol), each prepared in 30 mL of HOMOPIPES buffer (25 mM; pH 4.0). Nonlignified controls were stirred in HOMOPIPES buffer (25 mM; pH 4.0 with 3 mM $CaCl_2$). After stirring for a total of 48 h, lignified and nonlignified cell walls were collected on glass-fiber filters (1.2 μm porosity) and washed thoroughly with water followed by acetone and then air-dried. Experiments were replicated three times in a 2 × 2 factorial design with two levels of PME (0 or 2700 units) and two levels of coniferyl alcohol (0 or 200 mg) added per gram of cell wall.

Cell walls were analyzed by a colorimetric method for methanol (*15*) following hydrolysis with 0.5 M KOH at room temperature for 2 h. Acid-insoluble (Klason) lignin was determined by a two-stage

* To whom correspondence should be addressed: telephone (608) 890 0059; fax (608) 890 0076; e-mail jgrabber@wisc.edu.

10.1021/jf048799b
Published on Web 02/11/2005

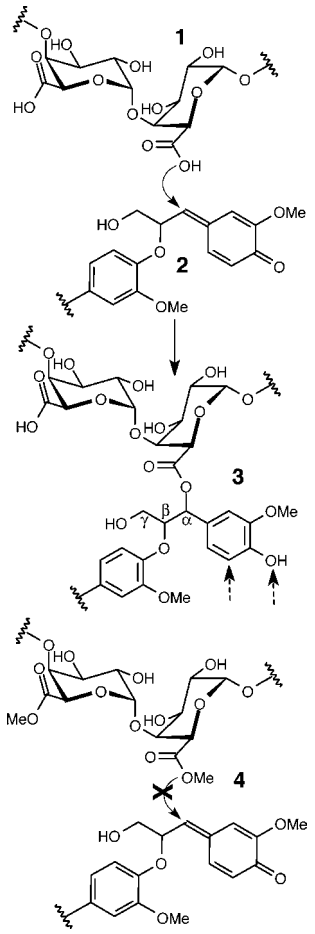

Figure 1. During cell wall lignification, polygalacturonic acid **1** can trap lignin quinone methide intermediates **2** to form benzyl uronate cross-links **3** between pectin and lignin. After cross-link formation, uronosyls may migrate from the α to the γ-position of the lignin side chain (*25*). Dashed arrows indicate potential coupling sites for further lignin polymerization. Methyl esterification of polygalacturonic acid blocks the formation of benzyl uronate cross-links **4**.

hydrolysis in 12 M H_2SO_4 at room temperature for 2 h followed by 1.6 M H_2SO_4 at 100 °C for 3 h (*16*). Acid hydrolysates from the second stage of the Klason procedure were analyzed for uronosyls by a colorimetric method (*17*) using galacturonic acid as a standard and with correction for hexose interference. Cell walls were suspended in acetate buffer (1% w/v; 20 mM; 40 °C; pH adjusted to 4.8 with NaOH; azide added to limit microbial growth) and degraded with Viscozyme L and Celluclast 1.5 L, each added at 40 μL/g cell wall (*7*). After 4 and 72 h of hydrolysis, cell wall residues were pelleted by centrifugation (2500 *g* for 10 min) and an aliquot of the supernatant was analyzed for uronosyls as described above and for total carbohydrate (*18*) using glucose as a standard. Data were subjected to analysis of variance and, unless noted otherwise in the text, all reported differences were significant at $P < 0.05$.

RESULTS AND DISCUSSION

Nonlignified cell walls isolated from maize cell suspensions typically contain about 110 mg/g of acid-soluble uronosyls and less than 10 mg/g of acid-insoluble lignin as measured by the Klason procedure (*10*, *14*, *19*). In the current study, cell walls were incubated in buffered NaCl solution at neutral pH with and without a commercial citrus PME to vary the degree of uronosyl methylesterification. This PME reportedly de-esterifies galacturonans in a block-wise manner without depolymerizing pectin (*20*). Cell walls were then suspended in pH 4 buffer with $CaCl_2$ and artificially lignified by slowly adding coniferyl alcohol and hydrogen peroxide. Lignification was carried out under acidic conditions to promote stable benzyl-uronate cross-linking (*21*). Ca^{2+} ions were added to help maintain peroxidase activity (*22*) and to stabilize pectins in cell walls (*23*). Nonlignified controls were incubated in pH 4 buffer with Ca^{2+}, but without the addition of lignin precursors.

Table 1. Concentration (mg/g of Cell Wall) of Klason Lignin (KL), Methanol (ME), Uronic Acids (UA), and Proportion of Methylated UA (Me-UA) in Nonlignified Maize Cell Walls Incubated with 0 or 2700 Units of Commercial Pectin Methylesterase (PME) and Then Artificially Lignified with 0 or 200 mg of Coniferyl Alcohol (CA) Added Per Gram of Cell Wall

treatment					
PME	CA	KL	Me	UA	Me-UA
0	0	*a*	9.4	100.0	0.57
2700	0	*a*	2.8	96.6	0.18
0	200	99.3	9.0	94.7	0.58
2700	200	115.9	3.4	87.3	0.23
analysis of variance					
PME		*b*	*b*	*b*	*b*
CA			*d*	*b*	*b*
PME × CA interaction			*b*	*d*	*c*
coefficient of variation (%)		3.4	3.4	2.1	5.7

[a] Not determined. [b] Significant at the 0.05 level of probability. [c] Significant at the 0.10 level of probability. [d] Not significant.

Nonlignified cell walls, not treated with PME, contained 9.4 mg/g of methanol and 100 mg/g of total uronosyls (galacturonic and glucuronic acids), suggesting a methyl ester level of 57%. Studies with maize coleoptiles indicate that roughly 80% of the uronosyls in primary cell walls are composed of partially methylated pectic galacturonic acids while the balance are nonesterified glucuronic acids associated with xylans (*3*). If cell walls from maize cell suspensions have a similar uronosyl make up, then about 70% of galacturonosyls were methyl esterified. Treatment with PME reduced methanol concentrations by 68% in nonlignified cell walls (**Table 1**), comparable to that reported for citrus pectin de-esterification by this enzyme preparation (*20*). We observed a slight 3% reduction ($P < 0.10$) in uronosyl concentrations of nonlignified cell walls treated with PME and incubated in acidic buffer. In preliminary studies (J. H. Grabber, unpublished), overnight incubation of maize cell walls in PME did not affect uronosyl concentrations and incubations of PME with citrus pectin indicated no polygalacturonase activity by reducing sugar assay. The manufacturer describes the PME as essentially free of polygalacturonase activity and none was detected in fractions isolated from this preparation (*20*). Nonetheless because of the large quantity of PME used, even trace contamination of polygalacturonase would lead to loss of uronosyls.

Although coniferyl alcohol was added to non-PME treated cell walls to form 165 mg/g of lignin, the Klason procedure indicated cell walls contained 99 mg/g of lignin. Under the acidic lignification conditions used in this study, about 40 mg/g of these Klason residues would be matrix components covalently attached to lignin (*13*), thus the actual efficiency of lignification was only about 35%. This is comparable to an earlier study where acidic pH promoted rapid inactivation of cell wall peroxidase (*13*). Under less acidic conditions, lignification efficiency typically exceeds 90% (*10*, *13*). Lignification of non-PME treated cell walls slightly reduced methanol and acid-soluble uronosyl concentrations (**Table 1**) without affecting the proportion of uronosyl methyl esters. This would be expected since polymerization of coniferyl alcohol into cell walls should

dilute the concentration of uronosyls and methanol without altering their relative proportions.

Pectin methylesterase treatment increased acid-insoluble lignin concentrations by 17% and tended ($P = 0.12$) to decrease acid-soluble uronosyl concentrations in lignified cell walls (**Table 1**), suggesting that demethylation of uronosyls enhanced benzyl ester cross-linking of pectins to lignin. Although Klason lignin residues are not expected to contain benzyl α-esters of galacturonic acid (*24*), migration of uronosyls from the α- to the γ-position of lignin side chains (*25*) may render benzyl-uronosyl esters less susceptible to acid hydrolysis as has been noted with other types of lignin γ-esters (*26*). Also, in contrast to non-PME treated cell walls, the methanol content and proportion of uronosyl methyl esters in PME-treated cell walls increased following lignification. Since lignification should not alter the methylation uronosyls, this data suggests that uronosyl concentrations were greater in lignified PME-treated cell walls than in nonlignified PME-treated cell walls, which could occur if benzyl ester cross-links reduced the extractability of galacturonans by acidic buffer.

Although these data provide good indirect evidence of greater benzyl uronate cross-linking in PME-treated cell walls, additional studies are needed to directly confirm the existence of such cross-links in our cell-wall model system and to assess whether uronosyl demethylation increases their abundance in cell walls and in Klason lignin residues. Direct analysis of benzyl uronate cross-linking is beyond the scope of this study, requiring the extension of NMR and solvolytic methods developed for lignin-carbohydrate complexes (*9*, *27*) to permit analysis of whole cell walls. A new method for dissolving whole cell walls for NMR or solvolytic analysis (*28*) should greatly facilitate the analysis of benzyl-uronate cross-links in future studies.

Cell walls were degraded with a mixture of Viscozyme and Celluclast, commercial enzyme preparations selected to provide a broad array of pectinase, cellulase, and xylanase activities. Viscozyme is particularly effective for degrading both partially and highly esterified polygalacturonic acids, but the former are degraded at a higher rate (*7*, *13*, *29*). As with cell walls digested by rumen microorganisms (*2*, *5*), the rate and extent of uronosyl release by this enzyme mixture outpaces that of most other cell wall sugars.

Among nonlignified treatments, a 68% reduction in methylation due to PME treatment had no effect on uronosyl release after 4 h but uronosyl release was increased by 8% after 72 h of enzymatic hydrolysis (**Table 2**). If 80% of maize uronosyls were galacturonic acid (*3*), then our results indicate that pectin hydrolysis by Viscozyme and Celluclast was rather insensitive to variations in polygalacturon methylation between 70% and 25%. This is in contrast to other work, indicating that hydrolysis of pectins by Viscozyme, as with other pectinases, was reduced by methylation (*8*, *29*). In our study, cell walls were incubated with sodium and calcium ions, which should facilitate gelling of pectin, particularly in PME-treated cell walls. Gelling impedes pectin hydrolysis (*30*) and this may have partially counteracted the beneficial effects of pectin demethylation. The overall release of total sugars from nonlignified cell walls was also not significantly influenced by PME treatment.

Lignification limited the release of uronosyls to a lesser degree than that observed for total sugars (**Table 2**), corroborating earlier work that pectin degradation is less affected by lignification than is cellulose or xylans (*2*, *5*, *31*). In contrast to nonlignified cell walls, reductions in methylation in lignified cell walls reduced uronosyl release by 55% after 4 h and by 7% after 72 h of enzymatic hydrolysis. Reductions in pectin methylation in lignified cell walls yielded only a modest 28 mg/g reduction ($P < 0.05$) in the release of total sugars at 4 h that was analogous to reductions in uronosyls released, again supporting earlier work indicating that the ease of pectin hydrolysis has little impact on the hydrolysis of other structural polysaccharides (*2*, *5*, *31*). Depressed release of uronosyls from lignified cell walls treated with PME clearly implicates benzyl-uronate cross-linking as a factor limiting pectin degradation but, as noted above, additional studies are needed to confirm their role in limiting pectin degradation in our cell wall model system as well as natural plant systems.

Table 2. Release of Uronosyls (%) and Total Sugars (mg/g of Cell Wall) from Maize Cell Walls during Incubation with Viscozyme and Celluclast, a Fungal Enzyme Mixture Containing Pectinase, Hemicellulase, and Cellulase Activity[a]

treatment		uronosyls		total sugars	
PME	CA	4 h	72 h	4 h	72 h
0	0	66.2	87.7	468.6	773.0
2700	0	68.7	95.0	467.5	768.0
0	200	48.4	87.8	157.8	519.1
2700	200	21.9	81.8	129.5	501.9
analysis of variance					
PME		*b*	*c*	*c*	*c*
CA		*b*	*b*	*b*	*b*
PME × CA interaction		*b*	*b*	*c*	*c*
coefficient of variation (%)		8.9	1.9	5.7	2.9

[a] Nonlignified maize cell walls were incubated with 0 or 2700 units of commercial pectin methylesterase (PME) and then artificially lignified with 0 or 200 mg of coniferyl alcohol (CA) added per gram of cell wall. [b] Significant at the 0.05 level of probability. [c] Not significant.

ABBREVIATIONS USED

PME, pectin methylesterase.

ACKNOWLEDGMENT

We thank Fachuang Lu for providing coniferyl alcohol and John Ralph for contributing to the preparation of this manuscript. Novo Nordisk Bioindustrials Inc generously provided Celluclast 1.5 L and Viscozyme L. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

LITERATURE CITED

(1) Wilson, W. D.; Barwick, J. M.; Lomax, J. A.; Jarvis, M. C.; Duncan, H. J. Lignified and non-lignified cell walls isolated from kale. *Plant Sci.* **1988**, *57*, 83−90.

(2) Titgemeyer, E. C.; Bourquin, L. D.; Fahey, G. C., Jr. Disappearance of cell wall monomeric components from fractions chemically isolated from alfalfa leaves and stems following *in-situ* ruminal digestion. *J. Sci. Food Agric.* **1992**, *58*, 451−463.

(3) Kim, J. B.; Carpita, N. C. Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol.* **1992**, *98*, 646−653.

(4) Carpita, N. C. Pectic polysaccharides of maize coleoptiles and proso millet cells in liquid culture. *Phytochemistry* **1989**, *28*, 121−125.

(5) Hatfield, R. D.; Weimer, P. J. Degradation characteristics of isolated and in situ cell wall lucerne pectic polysaccharides by mixed ruminal microbes. *J. Sci. Food Agric.* **1995**, *69*, 185−196.

(6) Femenia, A.; Garosi, P.; Roberts, K.; Waldron, K. W.; Selvendran, R. R.; Robertson, J. A. Tissue-related changes in methylesterification of pectic polysaccharides in cauliflower (*Brassica oleracea* L. Var. Botrytis) stems. *Planta* **1998**, *205*, 438−444.
(7) Grabber, J. H.; Hatfield, R. D.; Ralph, J. Diferulate cross-links impede the enzymatic degradation of nonlignified maize walls. *J. Sci. Food Agric.* **1998**, *77*, 193−200.
(8) Bonnin, E.; Le Goff, A.; Korner, R.; Vigouroux, J.; Roepstorff, P.; Thibault, J.-F. Hydrolysis of pectins with different degrees and patterns of methylation by the endopolygalacturonase of *Fusarium moniliforme*. *Biochem. Biophys. Acta* **2002**, *1596*, 83−94.
(9) Koshijima, T.; Watanabe, T. *Association between lignin and carbohydrates in wood and other plant tissues*; Springer-Verlag: Berlin, 2003; p 329.
(10) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.* **1998**, *46*, 2609−2614.
(11) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S. *p*-Hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability. *J. Agric. Food Chem.* **1997**, *45*, 2530−2532.
(12) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Severe inhibition of maize wall degradation by synthetic lignins formed with coniferaldehyde. *J. Sci. Food Agric.* **1998**, *78*, 81−87.
(13) Grabber, J. H.; Hatfield, R. D.; Ralph, J. Apoplastic pH and monolignol addition rate effects on lignin formation and cell wall degradability in maize. *J. Agric. Food. Chem.* **2003**, *51*, 4984−4989.
(14) Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T.; Pell, A. N. Dehydrogenation polymer-cell wall complexes as a model for lignified grass walls. *J. Agric. Food Chem.* **1996**, *44*, 1453−1459.
(15) Klavons, J. A.; Bennett, R. D. Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *J. Agric. Food Chem.* **1986**, *34*, 597−599.
(16) Hatfield, R. D.; Jung, H. G.; Ralph, J.; Buxton, D. R.; Weimer, P. J. A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. *J. Sci. Food Agric.* **1994**, *65*, 51−58.
(17) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.
(18) Dubois, M.; Giles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.
(19) Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry* **1995**, *40*, 1077−1082.
(20) Hotchkiss, A. T.; Savary, B. J.; Cameron, R. G.; Luzio, G. A.; Chau, H. K.; Fishman, M. L. Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. *J. Agric. Food Chem.* **2002**, *50*, 2931−2937.
(21) Sipilä, J.; Brunow, G. On the mechanism of formation of noncyclic benzyl ethers during lignin biosynthesis. Part 4. The reactions of a *β*-*O*-4-type quinone methide with carboxylic acids in the presence of phenols. The formation and the stability of benzyl esters between lignin and carbohydrates. *Holzforschung* **1991**, *45*, 9−14.
(22) Howes, B. D.; Feis, A.; Raimondi, L.; Indiani, C.; Smulevich, G. The critical role of the proximal calcium ion in the structural properties of horseradish peroxidase. *J. Biol. Chem.* **2001**, *276*, 40704−40711.
(23) Goldberg, R.; Morvan, C.; Jauneau, A.; Jarvis, M. C. Methylesterification, de-esterification and gelation of pectins in the primary cell wall. In *Pectins and pectinases*; Visser, J., Voragen, A. G. J., Eds.; Elsevier Science B. V.: New York, 1996; pp 151−172.
(24) Polakova, M.; Joniak, D.; Duris, M. Synthesis and acid-catalyzed hydrolysis of some 3-(4-methoxyphenyl)propyl glucuronates. *Collect. Czech. Chem. Commun.* **2000**, *65*, 1609−1618.
(25) Li, K.; Helm, R. F. Synthesis and rearrangement reactions of ester-linked lignin-carbohydrate model compounds. *J. Agric. Food Chem.* **1995**, *43*, 2098−2103.
(26) Grabber, J. H.; Quideau, S.; Ralph, J. *p*-Coumaroylated syringyl units in maize lignin: Implications for *β*-ether cleavage by thioacidolysis. *Phytochemistry* **1996**, *43*, 1189−1194.
(27) Xie, Y. M.; Yasuda, S.; Wu, H.; Liu, H. B. Analysis of the structure of lignin-carbohydrate complexes by the specific C-13 tracer method. *J. Wood Sci.* **2000**, *46*, 130−136.
(28) Lu, F.; Ralph, J. Nondegradative dissolution and acetylation of ball-milled plant cell walls: High-resolution solution-state NMR. *Plant J.* **2003**, *35*, 535−544.
(29) Evans, J. D.; Akin, D. E.; Foulk, J. A. Flax-retting by polygalacturonase-containing enzyme mixtures and effects on fiber properties. *J. Biotech.* **2002**, *97*, 223−231.
(30) Wehr, J. B.; Menzies, N. W.; Blamey, F. P. C. Model studies on the role of citrate, malate and pectin esterification on the enzymatic degradation of Al- and Ca-pectate gels: Possible implications for Al-tolerance. *Plant Physiol. Biochem.* **2003**, *41*, 1007−1010.
(31) Grabber, J. H.; Panciera, M. T.; Hatfield, R. D. Chemical composition and enzymatic degradability of xylem and non-xylem walls isolated from alfalfa internodes. *J. Agric. Food Chem.* **2002**, *50*, 2595−2600.

Received for review July 19, 2004. Revised manuscript received November 30, 2004. Accepted December 7, 2004.

JF048799B