J. Korean Soc. Food Nutr.
24(3), 389~395(1995)

Changes in Solution Properties of Pectins by Enzymatic Hydrolysis of Sidechains

Jae-Kwan Hwang† and Jozef L. Kokini*

Korea Food Research Institute, Kyonggi-do 463-420, Korea
**Dept. of Food Science, Rutgers University, NJ 08903, USA*

Abstract

The neutral sugar sidechains of apple pectins were hydrolyzed by commercial hemicellulases produced from *Aspergillus niger*, and the corresponding changes in solution viscosity were investigated in dilute ($c < c^*$) and concentrated ($c > c^*$) pectin solutions. Pectinase activity included in hemicellulases was removed by Epoxy-activated Sepharose 6B affinity chromatography using polygalacturonic acid as a ligand. Enzymatic hydrolysis of sidechains did not affect the specific viscosity of dilute (0.5%) pectin solutions, while viscosity significantly decreased in concentrated (2.0~6.0%) region. These results strongly suggest that the sidechains of pectins exist as an entangled state in concentrated solutions. It was also found that in the concentrated region the extent of viscosity reduction was dependent on pectin concentrations.

Key words : pectin, rheology, sidechains, hemicellulases

INTRODUCTION

Pectins consist of a linear α-(1,4)-linked D-galacturonic backbone, in which varying proportions of the galacturonic carboxyl groups are present as the methyl esters, and (1,2)-linked L-rhamnosyl residues are inserted at intervals (1). The sidechains, consisting mainly of D-galactose, L-arabinose, D-xylose, and less frequently D-mannose, L-fucose, D-glucose, and the rather rare sugars 2-O-methyl-D-xylose, 2-O-methyl-L-fucose and D-apiose, are covalently attached to the main backbone primarily through C-4 of the rhamnosyl residues, although substitution of the galacturonyl residues at either C-2 or C-3 is also found (2-4).

The sidechains of pectins include the sizable polymeric neutral sugar components such as arabinan, galactan and arabinogalactan of varying length (5,6). It has been reported that the sidechains of pectins amount to approximately 5~50% depending on the plant sources and the extraction methods (7). These neutral sugar sidechains are potentially significant in pectins, because there is evidence that such branches in synthetic polymers alter significantly physicochemical properties and in particular rheological properties primarily through their contribution to entanglement formation (8-10).

Hwang and Kokini (11) reported that the sidechains of apple pectins might be significantly entangled in concentrated solutions and thus conferred distinct rheological patterns depending on the degree of sidechains. The primary objective of the present study is to verify the intermolecular entanglements of pectins through the sidechains in concentrated solutions.

MATERIALS AND METHODS

Materials

Apple pectins were purchased from Sigma Chemical Co. (St. Louis, MO, USA), which were purified by copper precipitation and subsequent EDTA treatment (12). Hemicellulases (glycan hydrolase ; EC 3.2.1.) produced from *Aspergillus niger* were also obtained from Sigma Chemical Co. Epoxy-activated Sepharose 6B affinity chromatography medium was supplied from Pharmacia Fine Chemicals (Piscataway, NJ, USA).

†To whom all correspondence should be addressed

Concentration of hemicellulases

Ten grams of hemicellulases were dissolved in 200 ml of distilled, deionized water and centrifuged for 30min at 15,000rpm to precipitate impurities out. Then, 112.2g of $(NH_4)_2SO_4$ were added slowly to 80 % saturation at 4° C. After standing for 4h in a cold room, the solution was centrifuged for 30min at 15, 000rpm. The precipitate was dialyzed against 10mM sodium acetate buffer (pH 4.0) for affinity chromatography or 50mM sodium acetate buffer (pH 6.0) for rheological studies in a cold room for 24h.

Affinity chromatography (Removal of pectinases)

Preparations of Epoxy-activated Sepharose 6B affinity gel and the covalent attachment of polygalacturonic acid as a ligand were carried out according to the protocol supplied by Pharmacia Fine Chemicals (13). Epoxy-activated Sepharose 6B was swelled with 1L of distilled, deionized water on a sintered glass filter for 1h. Ligand solutions were prepared by dissolving 1.5g of polygalacturonic acid in 50ml of 0.1 M coupling borate buffer (pH 11.0). The hydrated affinity gel suspension was mixed with polygalacturonic ligand solution, pH of which was adjusted to 11.0 with 5N NaOH. After shaking for 24h at 35° C in the water bath, the remaining unreacted groups of the affinity gel were blocked with 100ml of 1M of ethanolamine for 4h at the room temperature. Then, the excessive ligand and ethanolamine were washed out thoroughly by washing with 0.1M coupling borate buffer (pH 11.0) followed alternatively by 0.1M borate buffer (pH 8.0) containing 0.5M NaCl and 0.1 M sodium acetate buffer (pH 4.0) containing 0.5M NaCl. The resulting affinity gel was suspended in 10 mM sodium acetate buffer (pH 4.0) and packed into a 1.6 × 20cm column.

The hemicellulase solution (1.0ml) concentrated by $(NH_4)_2SO_4$ precipitation was loaded to the affinity chromatography column previously equilibrated with 10mM sodium acetate buffer (pH 4.0), and the column was washed with 100ml of the same buffer on a flow rate of 15ml/h. The elutes were collected and dialyzed against 50mM sodium acetate buffer (pH 6.0) for rheological studies. Affinity gel was regenerated by washing with 0.1M sodium acetate buffer (pH 6.0) as suggested by Rexova-Benkova and Tibensky (14).

Preparation of substrates for pectinase assay

Polygalacturonic acid (Sigma Chemical Co.), employed both as substrates for the assay of pectinase activities (polygalacturonase and pectate lyase) and as a ligand of affinity chromatography, was purified by washing with 60% ethanol containing 5% (v/v) HCl and then 60% and 95% ethanol (15). Citrus pectins (Sigma Chemical Co.), employed as substrates for the assay of pectin lyase and pectinesterase, were purified by copper precipitation followed by EDTA treatment (12).

Characterization of pectins

Anhydrogalacturonic acid (AGA) content of pectins was determined by the m-hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (16). Methoxyl ($-OCH_3$) content of pectins was measured by the enzymatic method using alcohol oxidase as described by Klavons and Bennett (17), and the corresponding degree of esterification (DE) of pectins was calculated from AGA and methoxyl content (12,18). Gas chromatography was employed to identify and quantify the neutral sugar constituents of pectins as their alditol acetate derivatives (19,20).

The chemical composition of apple pectins is presented in Table 1 in conjunction with that of enzyme

Table 1. Chemical composition of polygalacturonic acid (PGA), apple and citrus pectins

Components	PGA		Apple[1]	Citrus[1]
	Before purification	After purification		
AGA[2] (% w/w)	94.64	98.91	71.13	73.65
Methoxyl (% w/w)	nd*	nd	8.06	10.17
DE[3] (%)	nd	nd	64.33	78.40
Neutral				
Sugars (% w/w)	6.24	1.32	12.07	5.76
Rhamnose	1.51	0.22	3.10	1.01
Arabinose	0.30	nd	0.80	1.71
Xylose	0.25	nd	2.89	0.10
Galactose	3.76	0.70	4.68	2.31
Glucose	0.42	0.40	0.60	0.63

* not detected
[1] Data from Hwang *et al.* (20)
[2] Anhydrogalacturonic acid
[3] Degree of esterification

substrates, i.e., polygalacturonic acid (PGA) and citrus pectins. It can be seen that original commercial polygalacturonic acid was significantly contaminated with neutral sugars, which may interfere with galacturonic acids released by polygalacturonase action during enzyme assay. However, after purification with 5% acid alcohol, approximately 99% purity of galacturonyl residues was observed with the absence of methoxyl content. It is most probable that the neutral sugar constituents of purified PGA are the integral part of pectin molecules.

Assay of pectinase activity

Polygalacturonase activity was assayed as follows (21): Mixtures of 0.2ml of 0.5% purified polygalacturonic acid in 50mM sodium acetate buffer (pH 4.5) and 0.2ml of hemicellulase solution were incubated for 30min at 37° C. Enzymatic reaction was terminated by adding 2ml of 0.1M borate buffer (pH 9.0). After adding 0.4ml of 1% 2-cyanoacetamide solution, the mixtures were heated for 10min at 100° C and cooled for 10min in a ice-water bath. Then, the absorbance was measured at 276nm using heated enzyme solution as a blank. The standard was obtained by 0~400nmoles of galacturonic acid in 0.2ml of 50 mM Na-acetate buffer (pH 4.5) by the same procedure as described above. A unit of polygalacturonase activity was defined as the amount that catalyzed the release of 1nmole of galacturonic acid per min at 35° C.

Polygalacturonase activity was also detected by viscometric measurements. Mixtures of 8ml of 1% purified polygalacturonic acid in 50mM sodium acetate buffer (pH 4.5) and 2ml of hemicellulase solutions were incubated for appropriate times. Then, 8ml of samples were applied to Cannon-Fenske capillary viscometer to measure viscosity changes.

Pectate and pectin lyase activities were determined spectrophotometrically by measuring the increment of absorbance at 235nm according to the method of McMillan and Phaff (22) and Albersheim (23), respectively. Pectinesterase activity was measured by the method of Thibault and Mercier (24).

Protein content of enzyme solution was colorimetrically determined by using a Bio-Rad (Richmond, CA, USA) protein assay kits prepared on the basis of Coomassie-blue dye-binding method (25). Bovine serum albumin was used as the standard for protein assay.

Rheological measurements

Dilute pectin solutions were prepared by dissolving 0.05g of apple pectins in 8ml of 50mM sodium acetate (pH 6.0) for 1h, in which 2ml of hemicellulase solution for a sample or 2ml of buffer for a control was added. Therefore, the final pectin concentration was kept constant at 0.5% (w/v). After incubating the mixtures for 12h at 30° C, 8ml of solution was transferred to the Cannon-Fenske capillary viscometer for the determination of specific viscosity (η_{sp}) as follows :

$$\eta_{sp}=(\eta-\eta_s)/\eta_s$$

where η is the viscosity of solution, and η_s is the viscosity of solvent.

Concentrated pectin solutions were prepared by dissolving the appropriate amount of apple pectins in 8ml of 50mM sodium acetate buffer (pH 6.0) for 2h, and then 2ml of enzyme solutions or 2ml of buffer was added to adjust the final pectin concentration. After incubating 12h at 30° C, shear viscosity was measured by a Rheometrics Fluid Spectrometer (Rheometrics Inc., Piscataway, NJ, USA). A cone and plate geometry with cone angle of 0.0196 radian and radius of 25mm was used for the measurements, and the gap size was 50 microns. The shear rates ranged from 0.1 to 100 sec^{-1} with 5 data points per log cycle. The experiments were conducted at 25±1° C. Steady shear viscosity is reported as a function of shear rate.

RESULTS AND DISCUSSION

Purification of hemicellulases

Hemicellulases are the group of hydrolytic enzymes responsible for the degradation of complex polysaccharides known as hemicelluloses (26). Thus, in this research the hemicellulosic sidechains of pectins were hydrolyzed enzymatically by using commercial hemicellulases produced from *Aspergillus niger*. However, it should be noted that hemicellulases employed should be free of pectinases which can depol-

ymerize the main backbone (polygalacturonase, pectin lyase and pectate lyase), or modify degree of esterification (pectinesterase) of pectins.

Ammonium sulfate ($(NH_4)_2SO_4$) precipitated hemicellulases used in this research possessed some polygalacturonase activity (specific activity : 0.35nmoles / μg protein / min), which can depolymerize the main backbone of pectin molecules. In contrast, pectin lyase, pectate lyase and pectinesterase activities were not detected. The results are consistent with the report of Thibault and Rouau (27). Since this research aims at elucidating exclusively the role of the sidechains of pectins, it is required to eliminate polygalacturonase activity so that the main backbone of pectin molecules remains intact after treating hemicellulases.

Fig. 1 shows that 80% $(NH_4)_2SO_4$ fractionated hemicellulases greatly reduced the specific viscosity of 1.0% polygalacturonic acid solutions due to its polygalacturonase activity, whereas hemicellulases after affinity chromatography did not affect the specific viscosity. This indicates that affinity chromatography using polygalacturonic acid as a ligand successfully eliminated polygalacturonase from hemicellulases.

Utilization of affinity chromatography for purifying pectinases was first introduced by Rexova-Benkova and Tibensky (14), who used a bifunctional reagent, ephichrohydrin (chloromethyloxirane), to produce the insoluble adsorbent matrix of pectic acid (polygalacturonic acid) by cross-linking. They reported that endo-polygalacturonase produced by *Aspergillus niger* exhibited biospecific affinity toward cross-linked pectic acid. The same procedure has been employed to separate pectinases from a variety of plant and microbial sources (28-30).

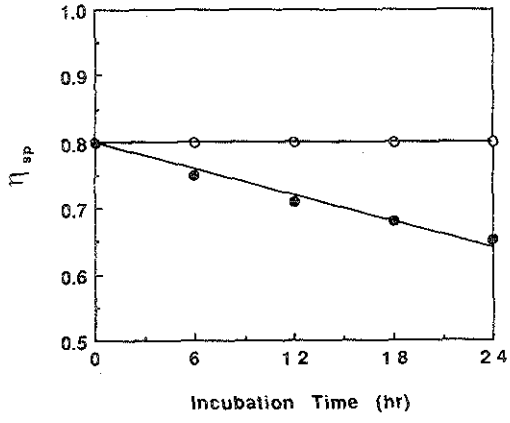

Fig. 1. Viscometric assay of polygalacturonase activity of hemicellulases.
○—○ : with purification (protein : 3.92μg/ml)
●—● : without purification (protein : 3.58μg/ml)

Rheological changes

Fig. 2 shows zero-shear viscosity (η_o) as a function of pectin concentration. It can be seen that η_o is drastically increased approximately above 1.0% concentration, indicating the transition from dilute to concentrated region at 1.0%. As the polymer concentration increases, a transition concentration ($c=c^*$) at which the polymer chain coils begin to overlap. In dilute region ($c<c^*$) molecules behave independently without contacting other molecules, whereas significant intermolecular entanglements through sidechains as well as backbone occur in concentrated region ($c>c^*$) (10,31,32). Therefore, above $c>c^*$ the viscosity increases very rapidly with increasing concentration, and at the same time the viscosity becomes more shear rate dependent (31). Consequently, in this research 0.5% and 2.0~6.0% were selected as dilute and concentrated regions, respectively, to understand the influence of sidechains of pectins on solution properties.

Fig. 3 shows the changes in η_{sp} of 0.50% apple pectin solution after treating hemicellulases with or without purification using affinity chromatography. It can be seen that hemicellulases without purification

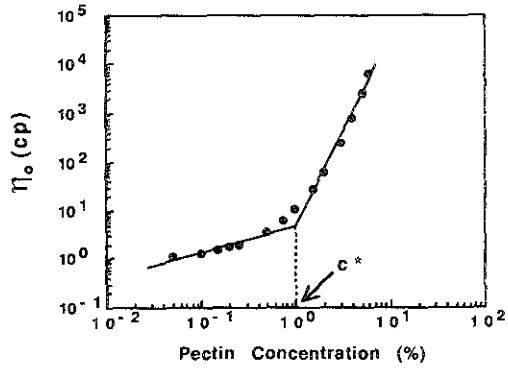

Fig. 2. Zero-shear viscosity(η_o) of apple pectins as a function of concentration.

reduced significantly η_{sp}, since they contained polygalacturonase activity depolymerizing the main backbone of pectins. However, η_{sp} was not nearly affected by hemicellulases undergone though affinity chromatography and thus devoid of polygalacturonase. These results suggest that debranching does not affect the solution viscosity in dilute apple pectin solution. A similar trend was observed in galactomannan which consists of mannan backbone and single galactose sidechains. McCleary *et al.* (33) demonstrated that the enzymatic removal of single galactose sidechains did not affect significantly the specific viscosity of locust bean and guar galactomannan in dilute solution.

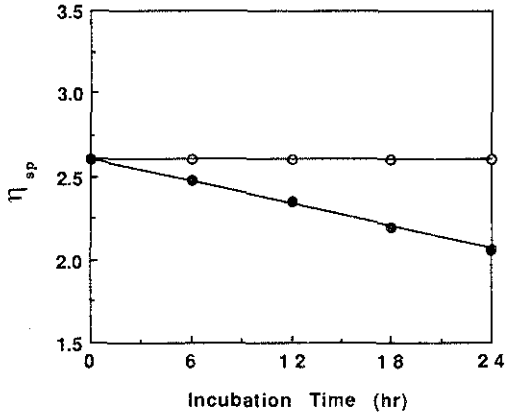

Fig. 3. Effect of hemicellulases on the specific viscosity (η_{sp}) of 0.5% apple pectin solution.
○—○: with purification (protein: 3.92μg/ml)
●—●: without purification (protein: 3.58μg/ml)

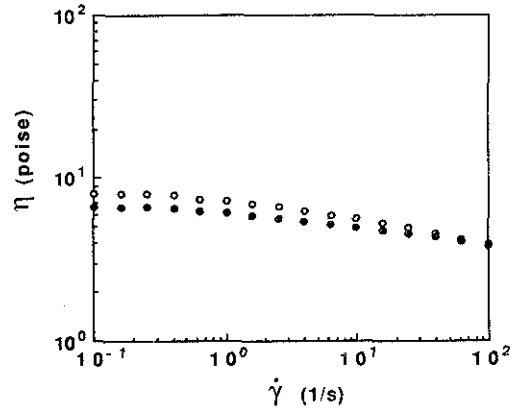

Fig. 4. Effect of purified hemicellulases on the viscosity of 4% apple pectin solution.
○—○: without enzyme
●—●: with enzyme (protein: 3.92μg/ml)

These results are not surprising, because sidechains as well as main backbone of polymers are not associated with molecular entanglements in dilute regions.

In contrast, Fig. 4 shows a significant decrease in shear viscosity by treating purified hemicellulases on 4% concentrated pectin solution. Since hemicellulases treated with affinity chromatography did not affect the main backbone of pectin molecules, the decrease in viscosity of 4.0% pectin solution is only attributable to disruption of the neutral sugar sidechains. These results strongly indicate that the sidechains are involved in entanglements of pectin molecules in concentrated region. If the sidechains are not associated with entanglements of pectin molecules in concentrated region, solution viscosity should be same even after applying hemicellulases. This explains why apple pectins exhibit distinct rheological properties depending on the branching degrees which affects the intermolecular entanglements (11).

In this research the effect of pectin concentration on rheological changes induced by enzymatic hydrolysis of sidechains was also investigated as a function of concentration. As shown in Fig. 5, the concentration effect can be divided into 3 distinct regions. At the region I of 2.0~4.0% pectin concentrations, a linear relationship existed between enzymatically induced viscosity reduction and pectin concentration. The results are consistent with Graessley's argument (34) that, as the polymer concentration increases, the number of entanglements increases correspondingly. At the

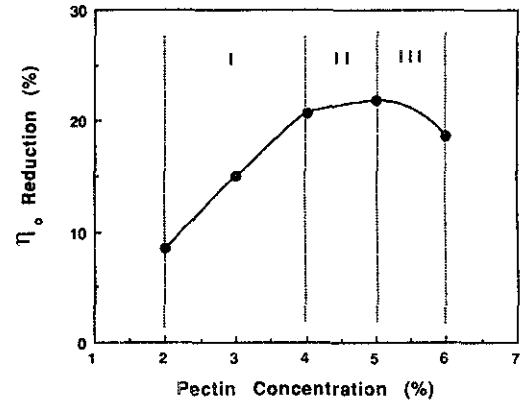

Fig. 5. The reduction of zero-shear viscosity (η_o) by enzymatic hydrolysis as a function of pectin concentration.

region II, however, the rate of viscosity reduction was significantly diminished with increasing concentration. This can be interpreted that the freedom of movement of the individual chain segments becomes restricted with increasing concentration and consequently, the rate to form new entanglements is reduced (34). At the region III, the viscosity reducing tendency was rather reversed. It is most probable that hemicellulases have the limited accessibility to sidechains because of high viscous system at 6.0% concentration. It was not successful to prepare pectins solutions above 6% concentration due to insufficient solubilization of pectins. Further studies are necessary to investigate in depth the entanglement density influenced by the sidechains as a function of pectin concentrations.

REFERENCES

1. John, M. A. and Dey, P. M. : Postharvest changes in fruit cell wall. *Adv. Food Res.*, **30**, 139 (1986)
2. Talmadge, K. W., Keegstra, K., Bauer, W. D. and Albersheim, P. : The structure of plant cell walls. *Plant Physiol.*, **51**, 158 (1973)
3. Selvendran, R. R., Stevens, B. J. H. and Du Pont, M. S. : Dietary fiber : chemistry, analysis' and properties. *Adv. Food Res.*, **31**, 117 (1987)
4. Bacic, A., Harris, P. J. and Stone, B. A. : Structure and function of plant cell walls. Ch. 8. In "*The Biochemistry of Plants*" Vol. 14, Preiss, J. (ed.), Academic Press, New York, p.309 (1988)
5. Aspinall, G. O. : Chemistry of cell wall polysaccharides. Ch. 12. In "*The Biochemistry of Plants*" Vol. 3, Press, J. (ed.), Academic Press, New York, p.480 (1980)
6. Dey, P. M. and Brinson, K. : Plant cell-walls. *Adv. Carbohydr. Chem. Biochem.*, **42**, 265 (1986)
7. Hwang, J., Pyun, Y. R. and Kokini, J. L. : Sidechains of pectins : some thoughts on their role in plant cell walls and foods. *Food Hydrocolloids*, **7**, 39 (1993)
8. Graessley, W. W. : Effect of long branches on the flow properties of polymers. *Accoun. Chem. Res.*, **10**, 332 (1977)
9. Roovers, J. : Branched polymers. In "*Encyclopedia of Polymer Science and Engineering*" Vol. 2, Klingsberg, A., Muldoon, J. and Salvatore, A. (eds.), John Wiley & Sons, New York, p.478 (1985)
10. Hwang, J. and Kokini, J. L. : Structure and rheological function of side branches of carbohydrate polymers. *J. Texture Studies*, **22**, 123 (1991)
11. Hwang, J. and Kokini, J. L. : Contribution of the side branches to rheological properties of pectins. *Carbohydrate Polymers*, **19**, 41 (1992)
12. Hwang, J., Roshdy, T. H., Kontominas, M. and Kokini, J. L. : Comparison of the effects of dialysis and metal precipitation on the physicochemical properties of apple pectins. *J. Food Sci.*, **57**, 1180 (1992)
13. Anoym. : Affinity Chromatography, Pharmacia. Uppsala, Sweden, p.27 (1988)
14. Rexova-Benkova, L. and Tibensky, V. : Selective purification of *Aspergillus niger* endopolygalacturonase by affinity chromatography on cross-linked pectic acid. *Biochim. Biophys. Acta*, **268**, 187 (1972)
15. Mikes, O. and Rexova-Benkova, L. : High-performance liquid chromatography of pectic enzymes. *Methods Enzymol.*, **161**, 385 (1988)
16. Blumenkrantz, N. and Asboe-Hansen, G. : New method for quantitative determination of uronic acid. *Anal. Biochem.*, **54**, 484 (1973)
17. Klavons, J. A. and Bennett, R. D. : Determination of methanol using alcohol oxidase and its application to methyl ester content of pectins. *J. Agric. Food Chem.*, **34**, 597 (1986)
18. Schultz, T. : Determination of the degree of esterification of pectin. *Meth. Carbohydr. Chem.*, **5**, 189 (1965)
19. Blakeney, A. D., Harris, P. J., Henry, R. J. and Stone, B. A. : A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.*, **113**, 291 (1983)
20. Hwang, J., Roshdy, T. H. and Kokini, J. L. : Effect of metal precipitation on the chemical composition of pectins. *Foods and Biotechnology*, **1**, 111 (1992)
21. Hwang, J. : Contribution of side branches of apple and tomato pectins to their rheological properties. *Ph. D. Dissertation*, Department of Food Science, Rutgers University, USA (1991)
22. McMillan, J. D. and Phaff, H. J. : Endopolygalacturonate lyase from *Clostridium multifermentans*. *Methods Enzymol.*, **8**, 632 (1966)
23. Albersheim, P. : Pectin lyase from fungi. *Methods Enzymol.*, **8**, 628 (1966)
24. Thibault, J. F. and Mercier, C. : *Aspergillus niger* endopolygalacturonase : 2. Characterization and some properties. *J. Food Biochem.*, **2**, 379 (1978)
25. Bradford, M. M. : A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248 (1978)
26. Dekker, R. F. H. and Richards, G. N. : Hemicellulases : their occurrence, purification, properties, and mode of action. *Adv. Carbohydr. Chem.*, **32**, 292 (1976)
27. Thibault, J. F. and Rouau, X. : Studies on enzymic hydrolysis of polysaccharides in sugar beet pulp. *Carbohydr. Polym.*, **13**, 1 (1990)
28. Rombouts, F. M., Wissenburg, A. K. and Pilnik, W. : Chromatographic separation of orange pectinesterase isoenzymes on pectates with different degrees of cross-linking. *J. Chromato.*, **168**, 151 (1979)
29. Tagawa, K. and Kaji, A. : Polygalacturonase from *Corticium rolfsii*. *Methods Enzymol.*, **161**, 361 (1988)
30. Schejter, A. and Marcus, L. : Isozymes of pectinesterase and polygalacturonase from *Botrytis cinerea Pers.* Me-

thods Enzymol., **161**, 366 (1988)
31. Ross-Murphy, S. B. : Rheological methods. In "*Biophysical Methods in Food Research*" Chan, H. M. S. (ed.), Blackwell Scientific Publication, Oxford, p.161 (1984)
32. Morris, E. R. and Ross-Murphy, S. B. : Chain flexibility of polysaccharides and glycoproteins from viscosity measurements. *Techniques in Carbohydrate Metabolism*, **B310**, 1 (1981)
33. McCleary, B. V., Amado, R., Waibel, R. and Neukom, H. : Effect of galactose content on the solution and interaction properties of guar and carob galactomannans. *Carbohydr. Res.*, **91**, 269 (1981)
34. Graessley, W. W. : The entanglement concept in polymer rheology. *Adv. Polym. Sci.*, **16**, 1 (1974)

(Received February 23, 1995)

펙틴 측쇄의 효소적 가수분해에 의한 용액특성의 변화

황재관† · 죠제프 엘 코키니*
한국식품개발연구원
*미국 럭거스대학교 식품과학과

요 약

사과펙틴의 측쇄를 hemicellulase를 이용하여 가수분해한 후, 묽은 용액(dilute solutions; $c<c^*$)과 진한 용액 (concentrated solutions; $c>c^*$)에서 용액점도의 변화를 관찰하였다. 효소에 포함되어 있는 polygalacturonase는 polygalacturonic acid를 ligand로 이용한 affinity chromatography로 제거하였다. 묽은 용액에서 측쇄의 가수분해는 점도에 큰 영향을 주지 않은 반면에, 진한 용액에서 측쇄를 가수분해한 경우에는 점도가 크게 감소하는 경향을 보였다. 이는 진한 용액 상태에서 펙틴의 측쇄가 분자간의 엉킴 (intermolecular entanglements)에 상당히 기여할 수 있음을 의미한다. 한편, 진한 용액(2~6%)에서 효소처리에 의한 점도감소는 펙틴의 농도의존성을 보였다.