Determination of Random- and Blockwise-type De-esterified Pectins by Capillary Zone Electrophoresis

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Capillary zone electrophoresis (CZE) was employed to determine the correlations between migration time and degree of esterification (DE) of pectinesterase-de-esterified pectins (PDPs) and alkaline-de-esterified pectins (ADPs) using 50 mM phosphate buffer (pH 6.5) as carrier electrolyte solution and 15 KV as applied voltage. Results showed that pectins with higher DEs exhibited shorter migration times. Linear correlation ($r = 0.995$) between migration time and DE of ADPs was observed, whereas down-curve correlation in PDPs was observed, regardless of the capillary length used (effective length, 30 and 60 cm). In addition, PDP appeared to migrate faster than ADP with the same DE under the same experimental conditions.

Keywords: *Degree of esterification; random-type de-esterified pectin; blockwise-type de-esterified pectin; capillary zone electrophoresis*

INTRODUCTION

Pectinesterase (PE) de-esterifies pectin substances that are constituents of the plant cell wall. The hydrolysis of methyl ester groups is catalyzed by this enzyme and produces a pectin with a lower degree of methylation during the ripening process of fruits and vegetables. Therefore, one of the major differences between pectic substances is their content of methyl esters, or degree of esterification (DE). The DE is defined as the number of esterified D-galacturonic acid residues × 100 + total number of D-galacturonic acid residues, which decreases with the increasing ripening stage. The DE of pectic substances is usually between 60 and 90%, depending upon the species, tissues, and maturity of the plants (*1*), whereas commercial pectic substances usually have DE values between 30 and 76% (*2*).

De-esterification of pectins is usually conducted with chemical reagents, such as acid and alkaline substances, and PE. Use of the former usually leads to the homogeneous (random) distribution of carboxylic acids in pectin molecules, whereas use of the latter results in the heterogeneous (blockwise) distribution in pectin molecules because of the strong binding effect between negatively charged pectins and positively charged PE (*3*, *4*) in pHs that are close to neutral. Determination of random- and blockwise-type de-esterification of pectin was previously performed by ion-exchange chromatography (IEC) (*3*) and high-performance liquid chromatography (*5*). However, the IEC method is lengthy and complex in determining the type of de-esterified pectins. Comparison of the activity coefficient of calcium ion in calcium pectate solutions prepared with various sources of PE has been used (*4*).

In determining the DE of pectic substances, Klavons and Bennett (*6*) developed an alcohol oxidase method which involved the oxidation of methanol, released from D-galacturonic acid by alkali hydrolysis, to formaldehyde, in the presence of oxidase. The quantity of formaldehyde formed was then determined by a spectrophotometer after reaction with a coloring reagent. Other methods such as borohydride (*7*), titration (*8*), and Fourier transform infrared (FTIR) (*9*) have also been used. However, the above-mentioned methods suffer from a major drawback: the lengthy time required for analysis; and a rapid and efficient method for determining the DE and the de-esterification type of pectin is urgently needed.

Capillary zone electrophoresis (CZE) is the most popular capillary electrophoresis (CE) technique. It uses the difference in electrophoretic mobility resulting from the variation in charge numbers and particle sizes between electrolytes under applied voltage. CE has the advantages of rapid separation with high resolution and high efficiency, requiring nanoliter levels of sample and media, comparing favorably to high-performance liquid chromatography (HPLC) (*10*−*15*). Under applied voltage, electrolytes and charged molecules are separated by the double effects of their own electrophoretic mobilities and the electroosmotic flow, which results from the electrical double layer in an uncoated fused silica capillary (*14*, *16*−*18*). Pectins with C_6 carboxyl groups in D-galacturonic acids are negatively charged in neutral pH, and the quantity of charge varies with the pectin DE (*2*). In previous research, alkaline-de-esterified pectins (ADPs) with various DEs were applied on a CZE, and the relationship between migration time and DE was found to be linear (linearity correlation, $r = 0.994$). Therefore, the determination of DE of ADPs by CZE appears to be possible (*19*).

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To determine the DEs of pectins de-esterified by alkaline and PE by CZE, pectins with various DEs were prepared with alkaline and PE from pea sprout. Then, both types of pectins were separated by CZE to observe the correlation between migration time and DE of pectin. Finally, the effect of capillary length on the migration time of pectins with various DEs was examined.

MATERIALS AND METHODS

Materials. Citrus pectin (DE = 65%) was a product of Sigma (St. Louis, MO). Methanol, 2-propanol, acetone, and sulfuric acid were purchased from J. T. Baker (Phillipsburg, NJ), and NaOH was from Merck (Darmstadt, Germany). The other chemicals were of analytical grade.

Preparation of Random-Type De-esterified Pectins with Various DEs. The DE of citrus pectin from Sigma was raised to 93% (*20*). Then, the pectin obtained (2%) was mixed thoroughly with an equal volume of various levels (0.03−0.1 N) of NaOH solutions (*3, 21*), and then slightly stirred in cold room (4 °C) for 30 min to randomly reduce the DE to 66, 56, 46, 37, 32, 22, 12, and 0%, as determined by the method described below, to prepare the alkaline-de-esterified pectins (ADPs). The pH value of the reaction mixtures was reduced to 4 by 1 N HCl to stop the de-esterification reaction. Linear correlation (*r*) between DE and NaOH level for a 30-min reaction time was found to be 0.998 (data not shown). Temperature (4 °C) was closely controlled throughout the reaction to avoid possible β-elimination and de-polymerization of pectin samples during alkali treatment (*21*), as determined by the absorption at 235 nm and by a Fractogel HW-65 (F) chromatography (data not shown), respectively. The ADPs thus obtained were dialyzed against deionized water (Milli-Q system, Millipore, Osaka, Japan) at room temperature (28 ± 2°C) for 12 h and then freeze-dried to make powders for the subsequent experiments.

Preparation of Blockwise-Type De-esterified Pectins with Various DEs. PE was extracted from pea (*Pisum sativum* L.) sprout with the method described by Jiang et al. (*19*). Pea sprouts (without endosperms), collected after spending 9 days in darkness, were blended first with three volumes of cold (4 °C) deionized water (Milli-Q system) and then with 0.01 M phosphate buffer solution (pH 8.0) to remove the soluble portions by filtration through a 6-layer piece of cheesecloth. Crude PE enzymes were extracted from the residues by using two vol 0.75 M NaCl, and then fractionated with 100% ammonium sulfate. After dialysis against 0.01 M phosphate buffer (pH 8.0), the obtained PE enzymes were applied on a CM-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) ion-exchange chromatograph to pool the fractions corresponding to peak 3 (PE 3). The dialyzed (against 0.01 M phosphate buffer, pH 8.0) pooled fractions were concentrated with a membrane concentrator (Amicon Co.; MWCO = 10 kDa) and then mixed well with 50% glycerol to 100 U/mL, as determined by the method described below, followed by refrigeration at −20 °C until use. Extraction and isolation of enzymes were conducted in a chilled room (4 °C ± 1 °C). PE 3 was used within one month after preparation.

Subsequently, 5 mL of the thus-prepared PE 3 solution (100 U/mL) was mixed well with 800 mL of 0.2 M NaCl/1% pectin with 93% DE at 30 °C ± 1 °C in a water bath with the pH value adjusted to 6.0 immediately before reaction. pH value of the mixture was maintained at 6.0 throughout the reaction with the addition of 0.3 N NaOH with a pH-stat (pH M83 Autocal pH meter, TTT 80 titrator, ABU80 autoburet (Radiometer Copenhagen Co., Copenhagen, Denmark). De-esterification was stopped by adjusting the reaction mixture to pH 4 using 1 N HCl (*22*), a thermal treatment in a boiling water bath given for 10 min after NaOH reached the desired amount (20−90 mL 0.3 N NaOH) to prepare the pectinesterase-de-esterified pectins (PDPs) with 72, 66, 65, 56, 52, 44, 38, 33, and 16% DE. A linear correlation (*r*) between pectin DE and volume of 0.3 N NaOH consumed was 0.997 (data not shown). PDPs thus obtained were freeze-dried after dialysis against 100 vol deionized water at 4°C.

Determination of PE Activity. PE activity was determined according to the method described by Lee and MacMillan (*23*) with minor modifications. Enzyme solution (1 mL) was mixed well with 15 mL of 0.1 M NaCl/0.5% citrus pectin solution at 30 °C ± 1 °C in a water bath with the pH adjusted to 6.0 immediately before assay. The activity of PE was measured by titrating the free protons dissociated from the free carboxyl groups formed by the PE activity. The volumes (mL) of 0.01 N NaOH used to maintain a pH level of 6.0 for the reaction solution at 30 °C were recorded within a reaction time of 5 min. One activity unit represents 1 μeq of the free carboxyl groups that were produced by the PE hydrolytic activity on the pectin substrate per min at 30 °C. An enzyme solution previously heated in boiling water for 5 min was used as a blank. Triplicate samples of each were analyzed twice.

Determination of Pectin. Pectin content of the obtained powders was determined with the method described by Blumenkrantz and Asboe-Hansen (*24*). Adequate volume (0.5 mL) of pectin solutions was mixed well with 3.0 mL of 0.0125 M Na-tetraborate solution (in c-sulfuric acid) in an iced bath and then heated in a boiling water bath for 5 min. After being cooled in an ice bath, the reaction mixture was mixed well with 0.05 mL of 0.15% *m*-phenylphenol/0.5% NaOH solution, and then allowed to rest for 5 min. Absorbance at 520 nm was recorded. Different levels (0−100 μg/mL) of D-galacturonic acid (Sigma, St. Louis, MO) were used to construct the standard curve for the calculation of pectin content in samples. Triplicate samples were each analyzed twice.

Determination of Pectin DE. DE of PDPs and ADPs was determined with the method described by Mizote et al. (*8*). Initially, a 2-g pectin sample was acidified in a mixture of 90 mL of distilled water, 10 mL of c-sulfuric acid, and 100 mL of 2-propanol with slight stirring by a magnetic stirrer for 15 min. Then, the pectin residues were obtained by filtration with Whatman No. 2 filter paper with the aid of an aspirator and were rinsed in a sequence of 300 mL of 65% 2-propanol, 200 mL of 2-propanol, and 50 mLof acetone to make pectin powders.

Subsequently, adequate powder (0.5 g) was obtained and moistened with 65% 2-propanol and then completely dissolved in 100 mL of distilled water. Volume (*a* mL) of 0.1 N NaOH solution was measured during the titration of pectin solution to pH 7.5. Pectin solution was obtained and mixed thoroughly with 30 mL of 0.1 N NaOH for 30 min, followed by the addition of 30 mL of 0.1 N sulfuric acid. Volume (*b* mL) of 0.1 N NaOH was measured during the titration of pectin solution to pH 7.5. The following calculation was used to determine the DE: DE (%) = $(b/a + b) \times 100\%$. Triplicate samples were each analyzed twice.

Apparatus and Electrophoretic Conditions. Electrophoretic separations were performed on a Beckman capillary electrophoresis instrument (P/ACE system 5500, Palo Alto, CA), equipped with a diode-array detector with a detective wavelength of 192 nm. Uncoated fused silica capillary tubing (effective length, 30 and 60 cm; inner diameter, 75 μm) purchased from Beckman (Palo Alto, CA) was rinsed successively with 0.1 N HCl and 0.1 N NaOH for 10 min each, then rinsed with deionized water and carrier electrolyte solution (50 mM phosphate buffer, pH 6.5) prior to use. Between analyses, the capillary was rinsed with 0. 1 N NaOH and subsequently with deionized water and carrier electrolyte solution for 3 min each. The separation column was kept at a constant temperature of 25.0 ± 0.1 °C by circulating a fluorocarbon liquid continuously through the cartridge, and the applied voltage was 15 kV. Sample introduction was performed using the pressure option for 5 s. Data collection was carried out with the Gold Chromatography data system version 8.0.

Pectins with various DEs were dissolved in deionized water to make a concentration of 5.0 mg/mL. Centrifugation (10000×*g*, 4 °C, 20 min) was performed to remove the precipitates from pectin solutions prior to use. Carrier electrolyte solution was also filtered through a 0.45-nm membrane filter prior to use.

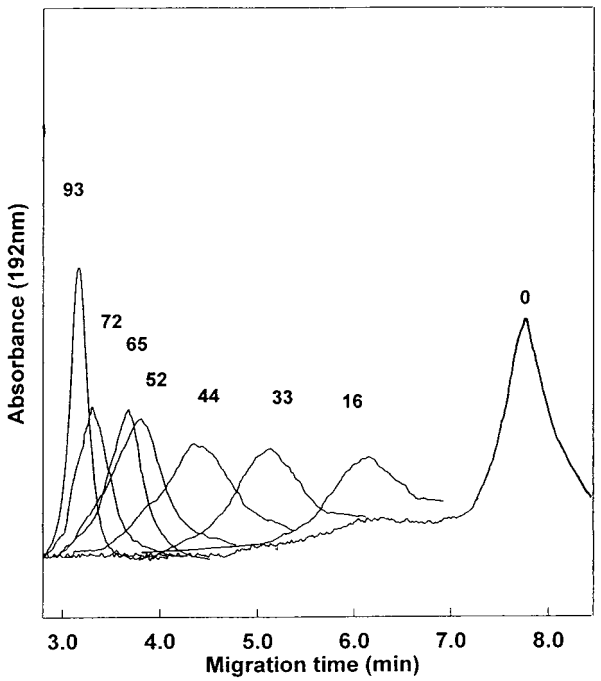

Figure 1. Capillary zone electrophoregrams of pectinestease-de-esterified pectins with various DEs. Pectin with 93% DE was treated with pectinesterase isolated from pea sprouts for various periods of times to prepare the pectins. Numbers represent the degree of esterification. Experimental conditions: pectin concentration, 5 mg/mL; column, uncoated fused silica column (effective tubing length, 30 cm; inner diameter, 75 μm); carrier electrolyte solution, 50 mM phosphate (pH 6.5); applied voltage, 15 kV; wavelength, 192 nm

Deionized water was obtained from a Milli-Q system (Millipore, Osaka, Japan). To investigate the effect of capillary length on the correlation between migration time and pectin DE, capillaries with effective lengths of 30 and 60 cm were used.

RESULTS AND DISCUSSION

Migration Time of Pectins with Various DEs. Figure 1 displays the migration times of PDPs using a capillary tubing with effective length of 30 cm. It was found that pectin with higher DE showed shorter migration time. The migration time of pectin with 93% DE was 3.2 min, whereas those with 72, 65, 52, 44, 33, 16, and 0% DE were 3.4, 3.7, 3.9, 4.4, 5.1, 6.2, and 7.8 min, respectively (Figure 1). It is well-known that pectin with higher DE possesses lower amounts of carboxylic acid groups in neutral solution, and thus, exhibits lower negative charges in a carrier electrolyte buffer with pH 6.5. Therefore, in the present study, pectin with higher DE migrated faster toward the cathode because of the presence of electroosmotic flow. Kuhn (*16*) and Kuhn and Monning (*17*) pointed out that the mobility of an electrolyte is dependent on the quantity of charge, molecular size, and molecular weight of the electrolyte in an electric field under a certain applied voltage. However, the stronger the negative charge of the electrolyte, the slower the observed mobility of an electrolyte toward the cathode because the electroosmotic flow is faster than the mobility of the electrolyte toward the anode (*16, 17*). Similar results were observed in the electrophoretograms when random-type de-esterified pectins with various DEs were applied on CZE (*19*). Furthermore, compared with the electrophoretograms of ADPs obtained by Jiang et al. (*19*), the peaks

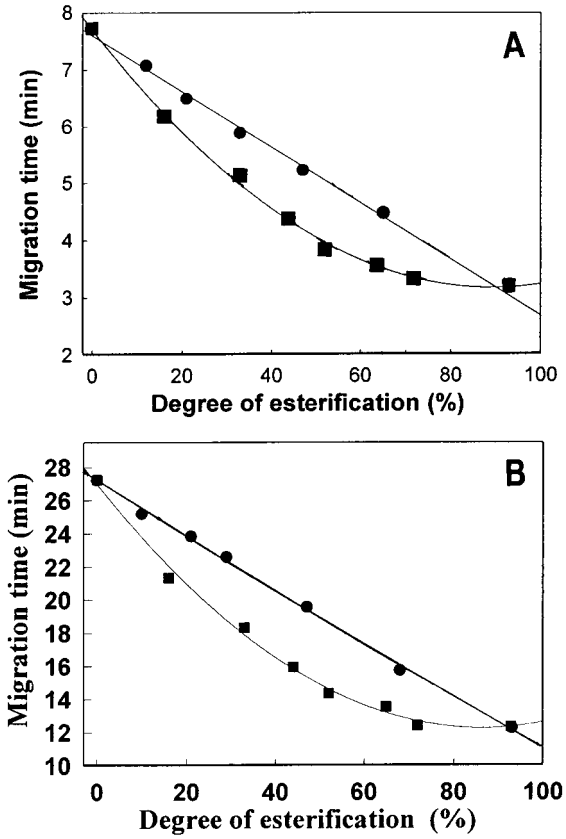

Figure 2. Correlations between migration time and pectin-esterase-de-esterified pectins (blockwise) (■) or alkali-de-esterified pectins (random) (●) with various DEs. Experimental conditions were the same as in Figure 1, except the effective tubing length was 30 cm (A) or 60 cm (B).

of PDPs in the present study appeared to be broader. In the present study, pectin with 93% DE was treated with PE 3 at pH 6.0 for various periods of time to prepare PDPs with various DEs. PE 3, like the other PEs from higher plants, is positively charged (pI = 9.3) and forms tight complexes with negatively charged high-methoxyl pectins (pK_a = 3.3) in PE−pectin reaction mixture at pH 6.0. Therefore, PE removes methoxyl groups in certain locations of pectin molecules and forms the heterogeneous de-esterification of pectins (*4, 25, 26*). Heri et al. (*3*) pointed out that PE formed heterogeneous distribution of negative charges in pectin molecules by comparing the D-galacturonic acid content and the averaged DE in the pooled fractions of alkaline-, acid-, and PE-de-esterified pectins separated by a DEAE-cellulose chromatography. Similar results were obtained by Schols et al. (*26*) by means of an ion-exchange chromatography. Therefore, the variation in the distribution of negative charges in pectin molecules could be responsible for the broadening effect of peak in PDP.

Correlation between Migration Time and DE of ADPs and PDPs. Figure 2 A shows the migration times of ADPs and PDPs with various DEs using a capillary tubing with an effective length of 30 cm. It was clear that ADPs showed a linear correlation (r = 0.995), but PDPs displayed a down-curve correlation between migration times and DEs, revealing that the difference in the distribution of charges in polysaccharides such as pectins might affect their electrophoretic migration behavior. A linear correlation (r = 0.994) between migration time and DE of ADPs was also observed by Jiang et al. (*19*). A possible explanation could be the

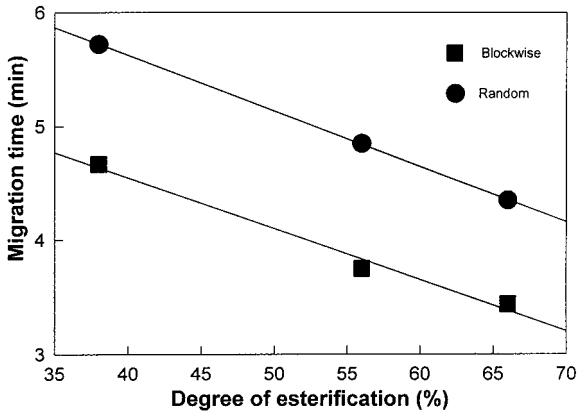

Figure 3. Correlations between migration time and pectinesterase-de-esterified pectins (blockwise) (■) or alkali-de-esterified pectins (random) (●) with various DEs. Experimental conditions were the same as those in Figure 1.

stronger repulsion between the heterogeneously negative-charged PDPs and the negatively charged inner surface of fused silica tubing, which resulted in the apparent reduction in friction between the inner surface of the tubing and analytes (*27*) and the accelerated migration toward the cathode. The high linearity between migration time and ADPs implies greater convenience in determining the DE of ADPs than in determining the DE of PDPs.

Increasing the capillary length to an effective length of 60 cm also displayed the similar correlation between migration time and DE (Figure 2B). The prolongation in capillary tubing increased remarkably the migration time of pectin, as reported by Peterson (*28*), Chang et al. (*29*), and Jiang et al. (*19*), as well as the resolution of electrolytes by reducing dissipation of the Joule heat and elevating the theoretical plate numbers (*29*). However, the major advantage of CZE with long capillary tubing is the big difference in migration times between pectins with 0% and 93% DE (Figure 2), suggesting that such CZE is more efficient in the DE determination of more pectin samples with different DEs. In addition, by comparing the results in Figure 2, differences in migration times between two types of pectins appeared to be more remarkable for pectins with DEs between 35% and 65%. It revealed that the utilization of CZE in distinguishing the type and determining the DE of pectins is more convenient when the DE of sample pectins is within this range.

ADPs and PDPs with 38%, 56%, and 66% DE were prepared, and their migration times were determined using a capillary tubing with an effective length of 30 cm. As shown in Figure 3, migration time of pectin decreased with increasing DE, regardless of the types of pectins, similar to the results in Figure 2. The migration time of blockwise-type de-esterified pectin was shorter than that of random-type pectin with the same DE. Pectins for food use generally had DEs between 30% and 76% (*2*), and therefore, these two correlations would be convenient for the determinations of both de-esterified type and the DE of pectins.

CONCLUSION

Down-curve correlation between migration time and DE of PDPs was determined, while high linear correlation was observed when ADPs were applied on CZE under the same experimental conditions. Reasons for the difference in correlation are unclear. It is probably due to the variation in the distribution of negative charges in the pectin molecules. Blockwise-type de-esterification forms heterogeneously high negative-charged areas in pectin molecules (*26*) in pH 6.0, resulting in the stronger repulsion between pectin molecules and inner surface of capillary tubing, and the accelerated migration of pectins toward the cathode. Moreover, de-esterified type and DE of pectins could be determined with the aid of a calibration curve constructed with the corresponding type of pectins with various DEs. The period of time required for CE analysis was less than 8 min in the present study, faster than the conventional methods such as ion-exchange chromatography. However, besides quantity of charge and charge distribution, molecular weight of charged organic substances such as pectin can also affect the migration time.

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