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Quantification of methyl ester content of pectin by pectinesterase

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Abstract. An alternative method for the determination of methyl ester content of pectin using the specific action of pectinesterase for hydrolyzing methyl ester group is described. The sodium hydroxide consumed during enzymatic reaction was used directly for the calculation of methyl ester content. This method was compared with that of acid-base titration. Degree of esterification determined by pectin esterase method is comparable to saponification method. This indicates that complete de-esterification is reached by the pectin esterase action. The detection limit of this method is 0.2 μmole methoxyl group in 10 ml pectin solution. This method provides a simple, rapid and selective procedure for measuring the methoxyl content of pectin.

Key words: Methyl ester content; Pectin; Pectinesterase.

Introduction

Pectin is the major component in the primary cell wall of plant. This hydrocolloids contain reactive, hydrolyzable substituent groups which can be monitored by different methods. An accurate determination of methoxyl group (OMe) is important to the study of colloidal functionality of pectin in plant biochemistry, and enable us to know better the structure of pectic substances, and how they influence the gelling properties and the solubility of pectin. Methods available to the OMe determination based upon different chemical principles have been developed.

Oxidation-reduction method is indirect (Doesburg, 1965; Laver and Wolfrom, 1962). It relies on the conversion of OMe to methyl iodide which is then transformed to methyl bromide and iodine bromide. Iodine bromide is oxidized to iodate, and iodate is reduced to iodine which is finally reduced further by thiosulfate. The *neutralization or saponification-titration method* (Schultz, 1965) is more direct. The free carboxyl groups are the group titrated. The OMe determination is then made by difference between carboxyl equivalents before and after saponification. This acid-base titration method is widely accepted as the standard method for evaluating pectins for commercial purposes (Food Chemical Codex, 1972). In addition to above, *colloid titration method* utilizing Cat-Floc was developed (Mizote *et al.*, 1975). The degree of esterification can effectively be determined by hydrolyzing the pectin with NaOH in the presense of Cat-Floc. Tibensky *et al.* (1963) based their method for the estimation of degree of esterification on the amount of bound Cu^{2+} ions before and after de-esterification. A widely used method for the determination of methoxyl content in pectin combines the saponification treatment and methanol determination. The released methanol was determined chemically based on the principle of its oxidation to formaldehyde which was determined colorimetrically after the formaldehyde is converted to colored solution (Nash, 1953; Sardesai and Provido, 1964; Wood and Siddiqui, 1971). The released methanol can also be determined by gas chromatography (Lee *et al.*, 1975; McFeeters and Armstromg, 1984). Physicochemical method using ^{13}C-NMR has also been devel-

oped for the estimation of the degree of methylation (Fishman *et al.*, 1984). Recently, pectin methylation was rapidly determined by Curie-point pyrolysis gas chromatography-mass spectrometry (Aries *et al.*, 1988). Basically all the methods mentioned above are catagorized as chemical and physical method. Biochemical method using pectin esterase (PE) to determine the methoxyl content was first presented by Hills *et al.* (1945). However, enzymatic method has not receive much attention during the past years. Recently a paper compared several methods for the determination of OMe suggested that enzymatic demethylation of pectin using PE destined for analytical purposes should be prefered (Kujawski and Tuszynski, 1987). The objective of this paper is to present a simple and specific procedure to analyze the methoxyl content of pectin enzymatically.

Materials and Methods

One commercial pectin preparation was examined: apple pectin (78% galacturonic acid, 7.4% methoxy content, lot 38F-0046, Sigma Chemical Co.). Two PE (EC 3.1.1.11) from tomato (lot 93F8110, Sigma) and citrus (lot 97F8055, Sigma) were investigated.

Theoretical Basis

During the enzymatic reaction, OMe at C_6 of galacturonic acid is hydrolyzed resulting in the formation of methanol and the carboxyl group left at the C_6. At pH 7.0, a pH far away from the pK=3.1 of polygalacturonic acid (Morvan *et al.*, 1979), both $-COO^-$ and H^+, in equal molarity, are in the majority, and only much less exists in the form of -COOH. The protons in the solution eventually are neutralized by the titrant NaOH. It is reasonable to assume that for one mole of NaOH used for titration must there be one mole of proton released from the carboxyl group of pectin. Therefore one mole of OMe of pectin hydrolyzed should be matched by one mole of carboxyl group. The reletionship between NaOH, proton, carboxyl group and OMe is the basis for our calculation of OMe in the pectin.

Procedure for the Determination of OMe by PE

Ten ml of 0.02% pectin solution in 0.1 N NaCl was poured into a beaker with a mechanical stirrer. The pectin solution is neutralized to pH 7.0 by adding NaOH. One to two units of PE, dissolving in distilled water freshly, was added to the neutralized pectin solution to initiate the enzymatic hydrolysis. End-point titration, set at pH 7.0, was carried out with a Metrohm autotitrator (614 impulsomat-E632 pH meter-665 Dosimat, Swiss) connected to a water bath with circulated water to keep the reaction temperature in the beaker at 37°C. The pectin de-esterification was regarded to be finished when pH underwent no change. This whole reaction usually takes about 15 min to finish. The amount of 0.01 N NaOH utilized was recorded. A blank determination is made without the addition of PE.

Calculation

The quantity of methyl-ester content of the sample in 10 ml of 0.02% pectin solution is calculated as the following.

OCH_3 content (mg)=ml of 0.01 N NaOH × 0.01 N × 31 (MW of OCH_3).

% of OCH_3=OCH_3 (mg) / polygalacturonic acid (mg) × 100

Interferences Study

The interference of PE by monosaccharides and other chemicals was studied. The reaction mixture contains 10 ml of 0.02% pectin solution in 0.1 M NaCl, 0.1 or 0.2 M of the tested monosaccharides, and 1 or 2 units of PE. Reaction proceeded at 37°C and the pH was kept at pH 7.0 with a Metrohm autotitrator as mentioned above. The de-esterification was regarded to be finished while pH underwent no change. One unit of PE activity is defined as the PE amount needed to release 1 μmole of proton per min at 37°C.

Acid-Base Titration Method

The polygalacturonic acid content and the degree of esterification of pectin were determined by acid-base titration (Food Chemical Codex, 1972). A 0.5 g amount of the apple pectin (Sigma) was weighed into a 250-ml beaker and moistened with 1 ml of 65% propan-2-ol, 100 ml of distilled water was added and the beaker was placed on a magnetic stirrer and stirred until all of the pectin has dissolved. The solution was then titrated with 0.1 N NaOH solution to pH 7.5, while stirring, and the volume of titrant consumed (a ml), which is corresponding to the free carboxyl groups, was noted. The stirring was continued, 30 ml of 0.1 N

NaOH solution was added and the beaker was covered and left for exactly 30 min. An amount of dilute H_2SO_4 equivalent to 30 ml of 0.1 N NaOH solution was added and, while stirring was continued, the mixture was titrated with 0.1 N NaOH solution to pH 7.5. The volume of titrant consumed again was noted (b ml). The degree of esterification (DE) was calculated as follows: DE (%)=100b/(a+b)

Uronic Acid Determination

Galacturonic acid was determined colorimetrically with carbazole reagent (McComb and McCready, 1952).

Results and Discussion

Determination of Degree of Esterification by Acid-Base Titration and Uronic Acid by Carbazole Reagent

The results of acid-base titration showed that the degree of esterification of apple pectin is 68.0%. Polygalacturonic acid is 77.2 μg in 100 μg pectin powder, and is 77.2% of original weight (Table 1). This is close to the 78% galacturonic acid content known from the label.

Unit of PE Activity Needed to Degrade Completely OMe of 10 ml of 0.2 mg/ml Pectin Solution

A substrate solution of 0.02% pectin in 0.1 M NaCl was tested for OMe content by using PE from citrus and tomato. The reaction was considered as complete when the pH underwent no change for 5 min. The results showes that one unit of both PE activity is enough to degrade OMe of 10 ml of 0.02% pectin solution (Fig. 1A). However the time needed for the completion of the reaction is different for these 2 enzymes (Fig. 1B). Tomato PE needs much longer reaction time and consumes less 0.01 N NaOH in order to degrade the same amount of pectin OMe in same volume of solution. About 510 μl and 560 μl of 0.01 N NaOH were used for tomato and citrus PE respectively.

Table 1. *OMe content and degree of esterification (DE) of pectin determined by acid-base titration and PE method*

	Acid-base	Sigma[c]	Tomato PE	Citrus PE
OMe, mg in 100 mg pectin		7.4	7.9	8.7
% of OMe[a]	11.1	9.6	10.2	11.2
DE[b], %	68.0	58.8	62.5	68.6

[a]Calculated from the OMe content and polygalacturonic acid content. Polygalacturonic acid is 77.2 mg in 100 mg pectin, determined by carbazole method (McComb and McCready, 1952).

[b]Calculated from the % of OMe content, assuming that 100% of OMe content is 16.32%.

[c]OMe content was provided on the label of pectin. DE was calculated based on the OMe content.

Calculation of the Methoxyl Content

(1) Utilizing tomato PE.

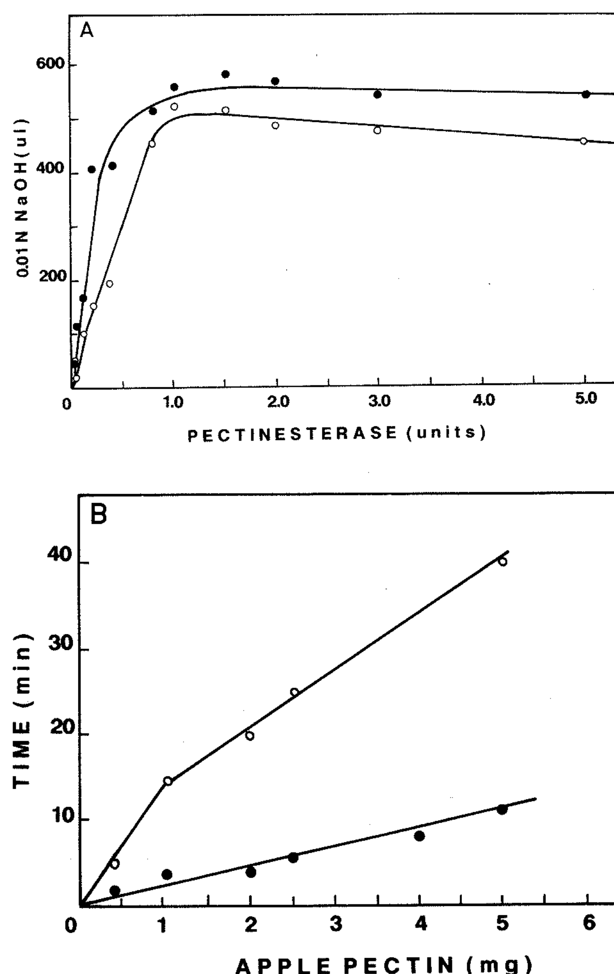

Fig. 1. A, Relationship between the consumption of 0.01 N NaOH and PE activity unit. Two mg of apple pectin in 0.1 M NaCl was used as the substrate for the hydrolysis by citrus PE (●) or tomato PE (○) in different activity unit. The reaction was held at pH 7.0, 37 °C for 20 min. B, Relationship between apple pectin and the time needed for the OMe hydrolysis. The reaction mixture contains 0.02% apple pectin in 0.1 N NaCl and one unit of citrus PE (●) or one unit of tomato PE (○).

OMe content: 0.51 ml NaOH solution × 0.01 N × 31 = 0.158 mg
polygalacturonic acid = 2 mg × 77.2/100 = 1.54 mg
OMe content (%): (0.158 mg / 1.54 mg) × 100 = 10.2%.

(2) Utilizing orange PE
OMe content: 0.56ml NaOH solution × 0.01 N × 31 = 0.173 mg
OMe content (%): (0.173 mg / 1.54 mg) × 100 = 11.2%

This was compared with 9.6% of OMe content of the original pectin (Table 1), and was comparable to the result obtained by acid-base titration method. This indicates that OMe hydrolysis of pectin by PE can be complete, therefore limiting enzymolysis short of complete de-esterification was not observed (Hill *et al.*, 1945). The larger consumption of NaOH by the citrus PE is unexplained at this moment. This could be due to no hindrance between the citrus PE and the apple pectin molecules so that the access of OMe by the PE faciliates the enzymatic hydrolysis. It also could be due to the different specificity of the enzyme.

Linearity Between Pectin Quantity and OMe Content

The reaction mixture contained 0.02% pectin in 0.1 M NaCl, and 1 unit of citrus PE or 2 units of tomato PE. The reaction was let to finish completely. The time needed for the hydrolysis of methyl ester groups was determined for citrus and tomato PE (Fig. 2). The linearity was good with high relative coefficient, however the reaction time was longer than 25 min if the pectin reaches 5 mg. The reaction catalyzed by 1 unit of citrus PE can be finished in about 10 min, and 2 units of tomato PE needs 13 min in order to hydrolyze the methyl ester groups (Fig. 3). Again the citrus PE consumes more 0.01 N NaOH for titration.

Interference Study

Interference study showed most of the monosaccharides in concentration up to 0.2 M have no influence on the PE activity. Galacturonic acid, the basic unit for the polygalacturonic acid, was found to have strong inhibition to the PE activity (Table 2). This seems much stronger than what have been reported for banana PE (Brady, 1976), papaya PE (Chang *et al.*, 1965) and jelly fig PE (Lin *et al.*, 1989).

Based on the results we consider that OMe content determination by PE action and NaOH titration has reduced the source of error when compared with the chemical methods. Enzymatic demethylation and direct methanol analysis has been proved to be a pre-

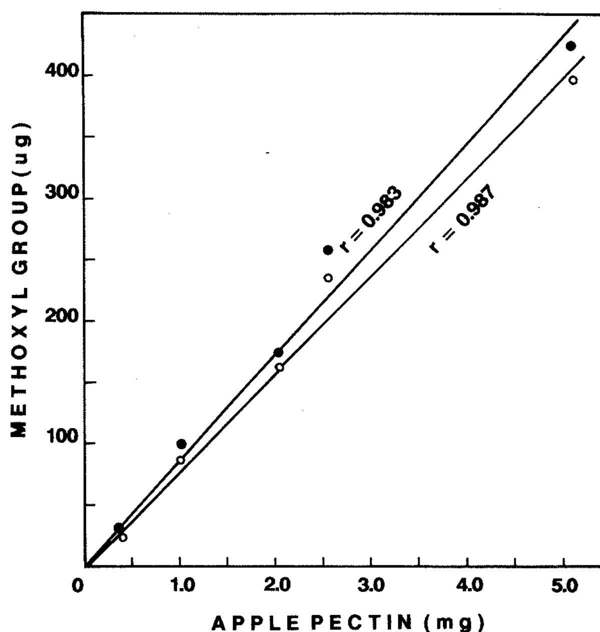

Fig. 2. Hydrolysis of OMe of 0.2% apple pectin in 0.1 M NaCl by one unit citrus PE (●) or two units tomato PE (○). The reaction condition same as that in Figure 1.

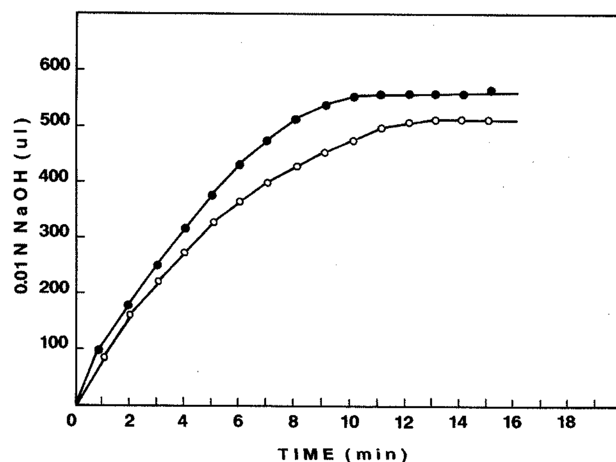

Fig. 3. Consumption of 0.01 N NaOH during the hydrolysis of 0.02% pectin in 0.1 N NaCl by one unit of citrus PE (●) or two units of tomato PE (○). Reaction condition same as that in Figure 1.

Table 2. *Interference of monosaccharides on PE activity*
Reaction conditions see "**Materials and Methods**".

Treatment	Final conc. M	Citrus PE activity[a] %	Tomato PE activity[a] %
Control	---	100	100
Ethanol	0.1	95.8	94.1
	0.2	99.1	100
Methanol	0.1	100	98.3
	0.2	95.4	99.6
Glucose	0.1	100	97.6
	0.2	97.5	97.2
Galactose	0.1	96.6	96.5
	0.2	100	100
Arabinose	0.1	99.1	96.5
	0.2	100	97.2
Galacturonic acid	0.1	0	0
	0.2	0	0
Sucrose	0.1	98.9	97.1
	0.2	100	97.1

[a]The PE activity in the reaction mixture without the added chemical was taken as 100%.

cise and selective one after Kujawski and Tuszynski (1987) compared the results obtained from four methods. Without the risk of methanol evaporation during the enzymatic reaction and without using gas chromatography and chemical methods for the methanol determination, PE method described in this paper is a very simple and rapid procedure. The procedure started from adding PE into the reaction mixture to the completion of hydrolysis only takes 10 to 15 min in the above stated condition. A method close to ours was presented originally by Hills *et al.* (1945) and has not been receiving attention during the years. Commercial availability of PE and the improvement of the instrument since then make PE method becomes very promising. Under suitable condition, as defined in this paper, 1-2 units of PE, can hydrolyze completely the OMe in 10 ml of 0.02% pectin solution within 15 min. Therefore the limitation of the application is the availability of how many sets of autotitrator. This method is also highly specific and selective without the danger of interference by most monosaccharides (Table 2). Detection limit of this PE method can be down to 20 μl of 0.01 N NaOH, this equals to 0.2 μmole of OCH_3 (0.02 ml $\times$ 0.01 N) in 10 ml solution. In other words, this is about 0.08 mg of high methoxyl pectin dissolved in 10 ml solution. Therefore this method is suitable for small sample and analytical purposes.

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利用果膠酯酶來決定果膠甲氧基之量

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本文描述果膠上甲氧基定量的另一方法乃是利用果膠酯酶水解甲氧基的專一性。酵素反應過程中所消耗的氫氧化鈉直接用來計算甲氧基之量。本法並與酸鹼滴定法相互比較。果膠酯酶法所獲得的酯化度與皀化方法所得相當。此一結果指出果膠酯酶可以達到果膠完全去酯的效果。本法的測定低限可達10毫升果膠液中僅含 0.2 μmole 甲氧基。此一方法提供了一個簡單、迅速與專一性之測量果膠上甲氧基之程序。