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Improvement of the selective depolymerization of pectic substances by chemical β-elimination in aqueous solution

T.P. Kravtchenko*, I. Arnould, A.G.J. Voragen & W. Pilnik

Department of Food Science, Agricultural University of Wageningen, P.O.B. 8129, 6700 EV Wageningen, The Netherlands

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The reaction of β-elimination which splits specifically the glycosidic linkages next to methoxylated galacturonic acid units without steric limitation may be used for the determination of the sequence of methyl esters along pectin molecules. However, due to competitive de-esterification, completeness of the reaction has never been achieved. In order to improve the extent of degradation, the influence of temperature, pH and buffer concentration on the β-elimination reaction in aqueous solutions has been investigated. Examination of kinetics revealed that any increase of temperature increases the rate of the β-elimination reaction more than that of the de-esterification. On the other hand, any decrease of pH decreases the rate of β-elimination but, much less than the rate of de-esterification. Buffer concentration plays an important role by controlling the pH through its buffering capacity as well as the availability of hydroxyl ions. Increasing the temperature up to 115°C and decreasing the pH down to 5·0 improved the splitting of the glycosidic bonds of a high methoxyl pectin backbone from 5·5 to 38% (53% of the glycosidic bonds contiguous to a methoxylated galacturonide unit). In spite of the appearance of various secondary reaction products, the absorbance at 235 nm can be used to estimate the extent of degradation.

INTRODUCTION

Pectin is mainly constituted of linear chains of 1,4-linked α-D-galacturonic acid units. The carboxyl groups of the galacturonic acid units are partially esterified with methanol. Although the average degree of methoxylation can be measured easily (Doner, 1986), the distribution of the methoxyl esters along the polygalacturonic acid chain is still largely unknown (Taylor, 1982). The development of a reaction splitting specifically and completely the glycosidic bonds adjacent to methoxylated or non-methoxylated galacturonic acid units would provide a unique tool to investigate the sequence of the methyl ester groups along the pectin chains.

Vollmert (1950) stated that glycosidic linkages between galacturonic acid units are broken during the alkaline de-esterification of pectins at room temperature. Kenner (1955), Neukom and Deuel (1958) and Whistler and BeMiller (1958) suggested that the splitting of pectin chains in alkaline solutions results from a base-catalyzed β-elimination reaction (also called 'β-de-alkoxylation'). Albersheim (1959) observed a similar degradation in hot neutral or weakly acidic conditions, which is an unusual phenomenon for most polysaccharides.

The β-elimination reaction proceeds on uronic acids which possess a glycosidic linkage on C-4 in the β position of the carboxyl group at C-5. According to this mechanism, the activated hydrogen atom on C-5 is removed by suitable proton-acceptors, leading to unstable, intermediary anions which are stabilized by losing the C—O linkage in the β position (Kiss, 1974). A double bond appears between C-4 and C-5 at the non-reducing end. Evidence from model compound studies (BeMiller and Kumari, 1972) suggests that the elimination does not take place by a concerted E2 process but by an E1cB anionic mechanism. In pectin, as the D-galacturonate residues are in the $^4C_1$ conformation (Deuel & Stutz, 1958; Rees & Wight, 1971), both substituents directly

* Present address: Centre de Recherche de Baupte, Sanofi Bio Industries, 50500 Carentan, France.

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involved in the β-elimination are in the axial position. Therefore, β-elimination of pectin is a diaxial process (Kiss, 1974), i.e. '*trans*-elimination'. This particular conformation makes the β-elimination reaction of pectin much easier and much faster than the *cis* (e,a) elimination of other natural polyuronates such as alginate (Albersheim *et al.*, 1960; Haug *et al.*, 1963; BeMiller & Kumari, 1972).

Albersheim (1959) suggested that the susceptibility of pectin to depolymerization by β-elimination is determined by the presence of its esters. Indeed, pectic acid was shown to be quite stable when similarly treated (Albersheim, 1959). Moreover, the β-elimination limit is closely related to the degree of methyl esterification of the pectin (Albersheim *et al.*, 1960; Rombouts & Thibault, 1986). The β-elimination reaction which depends on the presence of an ester group on galacturonic acid residues may thus be used for the specific depolymerization of the pectin backbone leading to the determination of the distribution of the acid groups (Henfrey, 1973).

This reaction performed in a 0·1 M Na-phosphate buffer at pH 6–6·8 has been used by several investigators (Barrett & Northcote, 1965; Stoddart *et al.*, 1967; de Vries *et al.*, 1983; Rouau & Thibault, 1984; Rombouts & Thibault, 1986). In such conditions, β-elimination is never complete because of a competitive reaction of de-esterification (Albersheim *et al.*, 1960; Rouau & Thibault, 1984). This partial depolymerization provided valuable information about the distribution of the side chains in pectins and led to the identification of 'hairy' and 'smooth' regions (Barrett & Northcote, 1965; Stoddart *et al.*, 1967) as well as valuable information about structural features of the neutral side chains of pectins (de Vries *et al.*, 1983; Kiyohara *et al.*, 1989) but was not sufficient to draw any conclusion about the distribution of methyl esters along the galacturonic acid chains.

β-elimination can also be catalyzed without de-esterification by enzymes called '*trans* eliminases' or 'lyases' (Pilnik & Rombouts, 1981) but complications due to steric limitation occur, e.g. neither pectin lyase nor pectate lyase can degrade pectin 'hairy regions' (de Vries *et al.*, 1982). Complete degradation is further limited by the fact that pectolytic enzymes require a complex binding subsite to recognize their catalytic site (Rexova-Benkova & Markovic, 1976), e.g. pectin lyase does not degrade esters of oligogalacturonides smaller than four units nor the two glycosidic bonds nearest the reducing end (Edstrom & Phaff, 1964), *exo*-pectate lyase from *Clostridium multifermentans* attacks the reducing end of the molecule where two units are cleaved at a time until any esterified group is encountered when reaction stops (McMillan *et al.*, 1964; Voragen, 1972). Moreover, pure enzymes are not always available. The chemical method which is not thought to be limited by steric hinderance (Rouau & Thibault, 1984) thus consitutes a prime method of obtaining the selective depolymerization of the pectin backbone.

Some factors of the chemical β-elimination reaction have already been studied. At pH 6·8, Albersheim *et al.* (1960) determined an approximate Q_{10} of 3·5 between 50 and 95 °C, demonstrating the strong temperature dependence of β-elimination. Keijbets and Pilnik (1974) stated that the cations Ca, Mg and K and the anions citrate, malate, phytate and chloride stimulate β-elimination at pH 6·1 and 100 °C. The rate of β-elimination increases with increasing pH because hydroxyl ions initiate the reaction (Neukom & Deuel, 1958) but the rate of de-esterification is also greatly increased. Doesburg and Grevers (1960) observed that, at pH 4·5–5·5 a marked decrease in the gelling power (i.e. degradation) of pectinic acid can be achieved without any appreciable decrease in the degree of esterification.

The β-elimination reaction can be followed by various methods. Since β-elimination results in a depolymerization, Albersheim *et al.* (1960) measured the drop of viscosity of pectin solutions. The decrease in molecular size can also be measured by HPSEC (Deckers *et al.*, 1986). However, as the depolymerization progresses, any new splitting of the polymer chain produces only a slight decrease in molecular size. It is thus difficult to quantify accurately the extent of degradation of highly degraded samples by evaluating the degree of polymerization. The periodate-thiobarbituric acid (TBA) test has been used (Albersheim *et al.*, 1960; Rombouts, 1972; Keijbets *et al.*, 1976) to detect unsaturated uronides produces by the β-elimination reaction. This test is very sensitive and specific but the molar extinction coefficient varies with the chain length (Voragen, 1972). Conjugation of the double bond with the carboxyl group on C-5 in the reaction products produce a strong absorption in UV at 235 nm.

In this paper, the effects of pH, temperature and buffer concentration have been investigated in order to reduce the unexpected de-esterification and thus increase the extent of degradation by chemical β-elimination.

EXPERIMENTAL

Pectin

An industrial 'rapid set' pectin from lemon peels (lemon A, Sanofi Bio Industries, France) with a uronide content of 84·2%, and a degree of methoxylation of 71·8% was used as substrate.

β-elimination

A fresh aqueous pectin solution (10 mg/ml) was mixed with an equal volume of the appropriate buffer solution. When necessary, the pH was corrected by the addition

of 0·1 M NaOH. Solutions were immediately divided into a series of sealed tubes and heated in a thermostated oil-bath for various times. Anhydrogalacturonic acid (AGA, MW = 176) content was determined in each final pectin solution using the automated *meta*-hydroxydiphenyl reaction (*m*hdp; Thibault, 1979).

Extent of degradation

The appearance of unsaturated uronide residues was determined by measuring the absorbance at 235 nm after appropriate dilution (20–60 fold). The extent of degradation (ED) is defined as the fraction of degradable glycosidic bonds that have been broken; degradable bonds being those contiguous to a methoxylated galacturonide unit. It was calculated using the following relation:

$$\text{ED}(\%) = \frac{A_{235} \times d/k}{\text{AGA} \times \text{DM}/100} \times 100$$

where A_{235} is the absorbance at 235 nm, d the dilution factor, k the molar extinction coefficient (liter/mol cm), AGA the anhydrogalacturonic acid (MW = 176) content (mol/liter) and DM the degree of methoxylation (%).

High performance size exclusion chromatography

HPSEC was performed using a series of Biogel TSK columns 40XL, 30XL and 20XL (300 × 7·5 mm) in combination with a Biogel TSK guard column (75 × 7·5 mm). Columns were eluted with 0·4 M Na-acetate buffer pH 3·0 at a flow rate of 0·8 ml/min and at 30°C. Detection was performed with a Showdex SE 61 refractive index detector at 40°C.

Enzymic β-elimination

Enzymic β-elimination was performed with an *endo*-pectin lyase (EC 4.2.2.10, type 2; van Houdenhoven, 1975) purified from a commercial enzyme preparation (Ultrazym, Gist Brocades, NL). Pectin solution (5 mg/ml) was incubated with pectin lyase (5 U/g pectin i.e. units of enzyme per gram of pectin, one unit being the amount of enzyme which splits 1 μmol of glycosidic bond per min) in 0·1 M Na-acetate buffer pH 4·2 at 30°C for 24 h.

RESULTS AND DISCUSSION

The effects of temperature, pH, buffer concentration and time of treatment on β-elimination have been investigated in order to optimize the reaction of β-elimination. In the light of published data (see Introduction) the following conditions were explored:

—temperature: 95, 105 and 115°C
—pH: 5·0, 5·4, 5·7 and 6·0

Na-citrate was chosen as the buffering agent from those tested by Keijbets and Pilnik (1974) because of its stimulating effect on β-elimination. Moreover, this buffer solution has proved to be perfectly stable (pH, color and UV spectrum) on heating at 115°C for 3 days. As the maintenance of the pH during the reaction depends on the strength of the buffer, kinetic studies would have required the use of highly concentrated citrate solutions. However, a high salt concentration would cause problems for further characterization of the degradation products and the authors have tried to reduce it as much as possible. Concentrations of 0·01, 0·05, 0·1 and 0·5 mol/liter were investigated.

All combinations of these parameters have been employed to reveal possible interactions. Thus, 48 different conditions were tested. Samples treated in 0·5 M buffer could not be analyzed because pectin precipitated very soon on heating.

Double bonds produced by the reaction of β-elimination have been quantified by reading the absorbance at 235 nm (Albersheim *et al.*, 1960). Edstrom and Phaff (1964) found a molar extinction coefficient of 5500 liters/mol cm for unsaturated methyl galacturonides but slightly different values were found for non-esterified unsaturated oligouronic acids (Nagel & Wilson, 1969; Voragen, 1972). The molar extinction coefficient of non-methoxylated unsaturated digalacturonic acid was measured and a value of 5610 liters/mol cm was found, which is not significantly different from that obtained by Edstrom and Phaff (1964). Thus, a molar extinction coefficient of 5500 liters/mol cm has been used for all the calculations in this study.

As first trials, degradations in the various conditions described above were performed during 24 h.

Extent of degradation after 24 h of treatment

The ED after 24 h (ED_{24}) increases clearly with the temperature (Fig. 1), confirming the findings of Albersheim *et al.* (1960) at lower temperatures. Depolymerization was most rapid at the highest temperature tested (i.e. 115°C). On average, ED_{24} increased by a factor of 3·0 between 95 and 105°C and by a factor of 1·8 between 105 and 115°C. At high buffer concentration, the positive effect of raising the temperature decreases with increasing pH. For instance, at a buffer concentration of 0·05 M, ED_{24} increases by a factor 5·8, 3·3, 2·5 and 2·1 between 95 and 105°C, at pH 5·0, 5·4, 5·7 and 6·0 respectively. In contrast, at lower buffer concentrations (0·01 M), ED_{24} always increases with increasing pH (Fig. 1).

The effect of the buffer concentration on ED_{24} is much more complex than implied above. Indeed, it is very dependent on other factors; especially, already pointed out above, the very clear interaction which occurs between the pH and the buffer concentration.

By raising the temperature up to 115°C, and

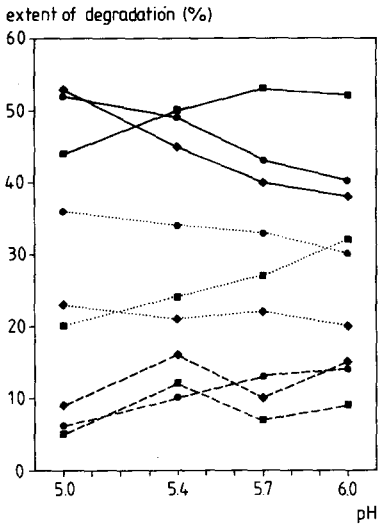

Fig. 1. Extent of degradation after 24 h of treatment at various pH values. Buffer concentrations: ■, 0·01 M; ●, 0·05 M; ♦, 0·1 M; temperature: — —, 95 °C; -----, 105 °C; ——, 115 °C. (Degradation is expressed as the percentage of split glycosiduronic linkages of all glycosiduronic acid linkages situated next to an esterified galacturonic acid unit.

decreasing the pH below 6, EDs higher than 50% were obtained after 24 h of treatment. For comparison, the same pectin sample was also treated in 0·1 M Na-phosphate buffer pH 6·8 at 80 °C for 24 h, and by pectin lyase (5 U/g, 24 h). EDs were 8 and 27% for chemical and enzymic β-elimination, respectively.

The effect of the various factors tested thus appears to be very complex. The authors have therefore examined the rates of depolymerization.

Rates of depolymerization

Reaction rates for the various sets of conditions have been observed for up to 24 h. All reaction rate curves display a similar general shape. They all tend to a plateau at which the reaction of β-elimination stops. It is likely that this limitation is due to the competitive reaction of de-esterification (Albersheim *et al.*, 1960). The height of the plateau can be interpreted as the ratio of the rate of de-esterification to the rate of β-elimination. This level decreases with increases in the ratio de-esterification to β-elimination. The kinetic curves also differ in their slope at the origin. At the beginning of the reaction, de-esterification has not removed enough methyl esters to limit the reaction of β-elimination. Therefore, it can be assumed that the slope at the origin is directly related to the rate of β-elimination.

When the temperature is raised from 95 to 115 °C, the β-elimination reaction is accelerated (Fig. 2). Although it is likely that the rate of de-esterification is also increased, the higher the temperature, the closer the plateau comes to 100%. Thus, any increase of the

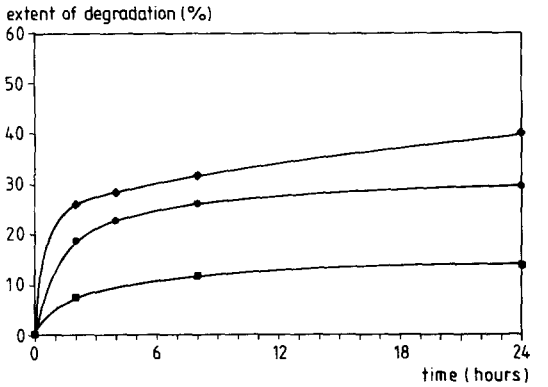

Fig. 2. Influence of the temperature on the rate of depolymerization by β-elimination in a 0·05 M Na-citrate buffer at pH 6·0: ■, 95 °C; ●, 105 °C; ♦, 115 °C.

temperature enhances β-elimination more than de-esterification.

When the pH is raised from 5 to 6, the reaction starts much faster (Fig. 3), confirming that β-elimination is favored by an increase of pH (Neukom & Deuel, 1958) but, the reaction also slows down much earlier. Indeed, de-esterification is much more favored by an increase of pH between 5 and 6 than β-elimination.

The buffer concentration does not exhibit such clear effects. It seems that depolymerization occurring at high pH values and low buffer concentrations is similar to that at low pH values and high buffer concentrations (Fig. 4). During the reaction, the pH of the solutions has been measured after cooling: after 24 h of treatment in 0·01 M buffer, the pH dropped to 4·4 when starting from 5·0 and to 4·8 when starting from 6·0, whereas it remained almost constant in 0·1 M buffer. Consequently, when the initial pH is high and the buffer concentration low, the pH-drop decreases the rate of de-esterification and the extent of degradation is much higher. On the other hand, when both the initial pH and the buffer concentration are low, the pH-drop slows down the β-elimination reaction. The drop in pH may be due to de-esterification of pectin which

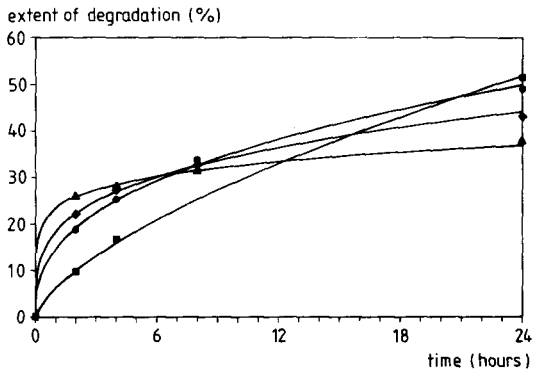

Fig. 3. Influence of the pH on the rate of depolymerization by β-elimination in a 0·05 M Na-citrate buffer at 115 °C: ■, pH 5·0; ●, pH 5·4; ♦, pH 5·7; △, pH 6·0.

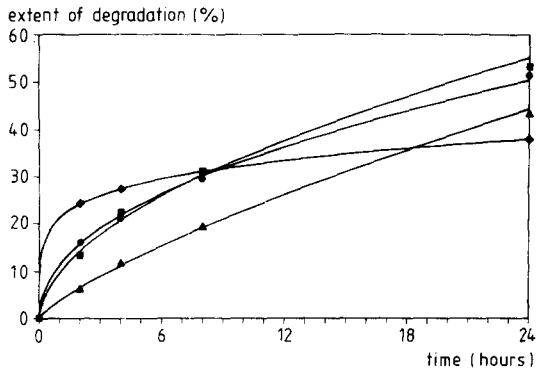

Fig. 4. Influence of the pH and the buffer concentration on the rate of depolymerization by β-elimination at 115°C: ■, 0·1 M, pH 5·0; ●, 0·01 M, pH 5·0; ♦, 0·1 M, pH 6·0; ▲, 0·01 M, pH 6·0.

releases free carboxylic acids. Besides, the drop in pH is smaller at low pH values which are less favorable to de-esterification.

Since salt concentration probably affects the rate of β-elimination (Keijbets & Pilnik, 1974) only slightly, it seems that buffer concentration only influences the extent of degradation by controlling the pH via the buffer capacity. However, the buffer concentration also influences the speed of de-esterification because neutral salts decrease the electrical potential of the molecules (Deuel & Stutz, 1958).

Examination of reaction rate curves shows that at low pH values and/or low buffer concentrations, even at high temperatures, the *trans*-elimination reaction is still proceeding after 24 h, suggesting that there are still suitable reaction sites available, i.e. esterified galacturonide residues.

Reliability of the calculated extent of degradation

Upon continued heating, the pectin solution turned a dark colour, possibly due to unsaturated compounds of increased conjugation (Albersheim *et al.*, 1960). Moreover, unsaturated oligogalacturonides produced by the β-elimination reaction are very unstable. Depending on the pH, their degradation produces 2-furoic acid, 5-formyl-2-furoic acid and a number of unidentified reaction products (Voragen *et al.*, 1988). Indeed, on prolonged heating, a second absorbance peak, probably due to these secondary reaction products, appears at 265–270 nm (Fig. 5). Henfrey (1973) suggested that the increase in absorbance at 235 nm may partly be due to an overlap of this second peak. Pectin depolymerization in certain conditions has also been monitored by HPSEC. It appears (Fig. 6) that samples treated with pectin lyase, i.e. in very mild conditions of temperature and pH elute much later on HPSEC than samples of similar ED degraded by chemical β-elimination. Even if a difference in elution time at the top of the peak does not imply a large difference in the degree of polymerization, it seems that the appearance of secondary reaction products leads to an over-estimation of the true extent of degradation. On the other hand, Voragen *et al.* (1988) showed that the unsaturated trimer and dimer are degraded to the corresponding saturated dimer and monomer respectively, next to the other reaction products. The authors observed that the absorbance at 235 nm of a solution of unsaturated digalacturonide decreases on heating at 115°C and pH 5. Disappearance of 4,5-unsaturated units leads to an under-estimation of the true extent of degradation. Thus, it is very unlikely that the ED calculated on the basis of the absorbance at 235 nm accurately measures the real extent of degradation. Nevertheless, calculated ED can be accepted as a good indicator of the extent of reaction since both secondary reactions which may alter its validity are closely related to the amount of unsaturated galacturonide units. This assessment is demonstrated by the

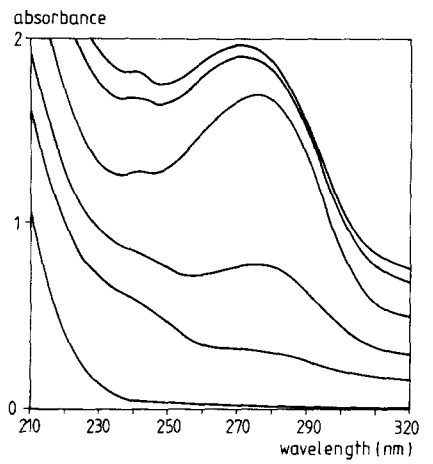

Fig. 5. Changes in the UV spectrum of the reaction products (diluted 30 times) of chemical β-elimination after 0, 4, 8, 24, 48 and 72 h of treatment at pH 5 and 115°C.

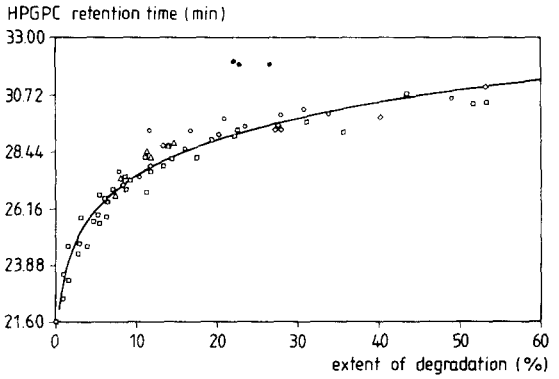

Fig. 6. Relationship between the extent of degradation calculated on the basis of the absorbance at 235 nm and the HPSEC elution time at the top of the peak for samples treated in various conditions of temperature, buffer concentration and pH: □, pH 5·0; ○, pH 5·4; ◇, pH 5·7; △, pH 6·0; ●, sample treated with pectin lyase.

clear correlation existing between ED and HPSEC elution time of chemically treated samples (Fig. 6).

CONCLUSION

Systematic study of the rates of reaction between 85 and 115 °C and from pH 5·0 to 6·0 in buffers of various concentrations showed that both β-elimination and competitive de-esterification are favored by increasing the temperature and increasing the pH. However, any increase of temperature favors β-elimination more than de-esterification and any increase of pH favors de-esterification more than β-elimination. Thus, to increase the extent of degradation, it is advantageous to increase the temperature and decrease the pH. The buffer concentration must be high enough to avoid any change of the pH during the reaction.

Within the conditions tested in this study, it is possible to recommend that the β-elimination reaction is carried out in 0·1 M Na-citrate buffer pH 5·0 at 115 °C. In such conditions it is possible to split 38% of the glycosidic bonds of a high methoxyl pectin galacturonan chain after 24 h of treatment. For comparison, the same pectin sample treated in the usual conditions (80 °C, pH 6·8, 24 h) could only be degraded up to 5·5%.

The rate of reaction in such conditions showed that the reaction can still be improved by prolonging the reaction time. Moreover, it seems that high ionic strength stimulates the reaction of de-esterification (Deuel & Stutz, 1958). The extent of degradation might be increased by the use of lower concentrations of a buffer of high capacity at pH 5·0.

This technique has been applied for the specific depolymerization of three industrial high methoxyl pectins differing in physical behavior. The results obtained will be presented in a following paper.

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