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Distribution of Methoxyl Groups in Pectins

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SUMMARY

To study the distribution of the methoxyl groups, pectins (mainly from lemon peels) have been degraded extensively by purified pectin lyase. The resulting fragments were fractionated by gel permeation chromatography and high-pressure liquid chromatography (ion exchange). The chromatograms of the pectins were compared to those of 'transesterified' pectins in which a random distribution of the methoxyl groups is assumed. The results indicate that differences exist between natural pectins and transesterified pectins. In native pectins more isolated non-esterified galacturonate residues are present. The oligomer pattern (distribution of degree of polymerization) of the pectin lyase degraded pectins was also found to be different for native and transesterified pectins.

INTRODUCTION

The problem of the distribution of methoxyl groups has received considerable attention in recent years (Tuerena *et al.*, 1982, 1984; de Vries *et al.*, 1983). The problem is interesting from a technological point of view (structure–function relation of commercial pectins) and from a plant-physiological point of view (function of pectic substances in the cell walls). Much attention has been paid to the impact of pectinesterase action on the distribution of methoxyl groups in pectins (Kohn *et al.*, 1985). It is generally assumed that plant pectinesterases result in a blockwise distribution of methoxyl groups, whereas mould

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enzymes do not, although a microbial pectinesterase has been found that brings about a blockwise distribution (Markovic & Kohn, 1984). This conclusion is mainly based on Ca^{2+}-binding experiments.

Methods of studying the distribution of methoxyl groups have been reviewed by Taylor (1982). Fractionation must be applied to investigate the intermolecular distribution, whereas the elucidation of the intramolecular distribution requires degradation of the molecules. Unfortunately, no reliable degradation method exists which can split specifically glycosidic linkages from esterified galacturonate residues. Such bonds can be split by β-elimination, but the reaction does not proceed to completeness and is accompanied by de-esterification. Tuerena *et al.* (1982, 1984) developed a method based on enzymatic degradation of glycolated pectins. This method may, however, result in an overestimation of the amount of 'blocks', because an isolated non-glycolated galacturonate residue is probably protected from enzymatic attack by the surrounding glycolated galacturonate residues.

De Vries *et al.* (1983) used purified pectin lyase (capable of degrading high methoxyl pectins) and pectate lyase (degrades only low methoxyl pectins). In carefully extracted apple pectic substances the intramolecular distribution could not be distinguished from a random one.

The present paper reports on the degradation of pectins (mainly from lemon, but also from apple, orange and lime) with purified pectin lyase. The degradation products are fractionated by gel filtration and by an HPLC method. The results yield not only information about the fine structure of the pectins but also about the mode of attack of the enzyme.

MATERIALS AND METHODS

Materials

Pectins used were samples from The Copenhagen Pectin Factory, Denmark (GENU Pectins), Grindsted, Denmark (Mexpectin). Orange pectins were obtained by acid extraction from fresh orange peel (3–8 h, 70–80°C, pH ~ 2, H_2SO_4). Apple pectin was from Unipectine, France. The pectins have not been subject to de-esterifying procedures during manufacturing. Chemicals used were of analytical grade.

'Transesterified' pectins were obtained by esterification of GENU Pectin AA in cold acidified absolute methanol for 2 weeks as described by Heri *et al.* (1961) and subsequent de-esterification in cold aqueous solution by addition of calculated amounts of 0·1 N NaOH (24 h, 3°C).

Methods

Analytical methods

The anhydrouronic acid (AUA, $M_w = 176$) content of pectin fractions was determined by carbazole sulphuric acid assay (measuring A_{520} in a mixture of 2 ml 0·1% carbazole in H_2SO_4 and 1 ml of pectin or degraded pectin 30 min after mixing).

The degree of esterification was determined by titration with 0·1 M KOH of the methanol/water washed pectin before and after alkaline de-esterification.

Gel filtration

A sample of (degraded) pectin (3–8 mg in 2 ml buffer) was applied to a Sephacryl S-500 (Pharmacia) column (80 × 2·5 cm) and eluted with 0·1 M Na-phosphate buffer, pH = 5·4 containing 0·1% hexametaphosphate. The flow rate (0·25 ml min^{-1}) was controlled by an LKB peristaltic pump. The whole procedure was conducted at room temperature. The galacturonic acid recoveries of the experiments were found to be about 100%.

Enzymatic degradation

Pectin lyase (EC 4.2.2.10) was purified from the commercial preparation Ultrazyme 100 according to the procedure of van Houdenhoven (1975). However, in this procedure the last step (a Ca-phosphate column) was replaced by chromatography on a crosslinked pectate column (Rombouts *et al.*, 1978) to bind residual polygalacturonase activity. The pectin lyase was of type 2 (van Houdenhoven, 1975); the preparation was devoid of activity of pectate lyase, polygalacturonase and pectinesterase.

The extent of degradation (% of bonds broken) was determined spectrophotometrically at 235 nm assuming $\varepsilon_{235} = 5500$ M^{-1} cm^{-1} (Edstrom & Phaff, 1964) for the unsaturated esterified product.

Enzyme reaction conditions were as follows: 2 mg ml^{-1} pectin in McIlvaine buffer pH = 6·0 containing 0·02% hexametaphosphate and 1·5 units ml^{-1} enzyme (1 unit releases 1 μmol min^{-1} unsaturated products in a standard assay (van Houdenhoven, 1975)) at room temperature (25°C) for 6 h.

High-pressure liquid chromatography

A Waters HPLC pump (P590) and injector were used equipped with a Waters fixed wavelength UV detector (at 254 nm) and a recording integrator (Waters QA1). A 250 × 4·6 mm internal diameter LiChrosorb 10 NH_2 column was eluted with 0·1 M Na-acetate buffers of varying pH (Voragen *et al.*, 1982). The amounts of oligomers (after cold alkaline de-esterification of the degraded pectins) were quantified using unsaturated digalacturonic acid as standards.

RESULTS AND DISCUSSION

Figure 1 shows a typical gel filtration pattern of a pectin before and after pectin lyase degradation. The small front of the peak in the case of degraded pectin probably represents the neutral sugar rich parts of the molecules, the 'hairy regions' (de Vries *et al.*, 1982). When applied to a gel filtration column containing Biogel P2, less than 5% of the uronic acid material appears in the 'void volume'. This implies that all the oligomers have a degree of polymerization (DP) of less than about 15. Figure 1 indicates that all or almost all molecules have been degraded by the enzyme: all the degraded pectin molecules appear to elute in the 'included volume'. It seems, therefore, reasonable to assume that all the molecules have a degree of esterification of more than about 35%, because the enzyme cannot degrade low-ester pectins. It can be concluded that the intermolecular distribution of the degree of esterification does not cover the whole spectrum from 0 to 100%. It may well resemble the distribution in apple pectic substances in which 90% of the molecules have a DE between 65 and 85 (de Vries *et al.*, 1983). Anger & Dongowski (1984) found two peaks in commercial pectins on ion exchange chromatography; it must be said, however, that in ion exchange chromatography of pectins the molecular weight is a factor of considerable importance. An interesting example of intermolecular distribution is tomato pectin, as Huber &

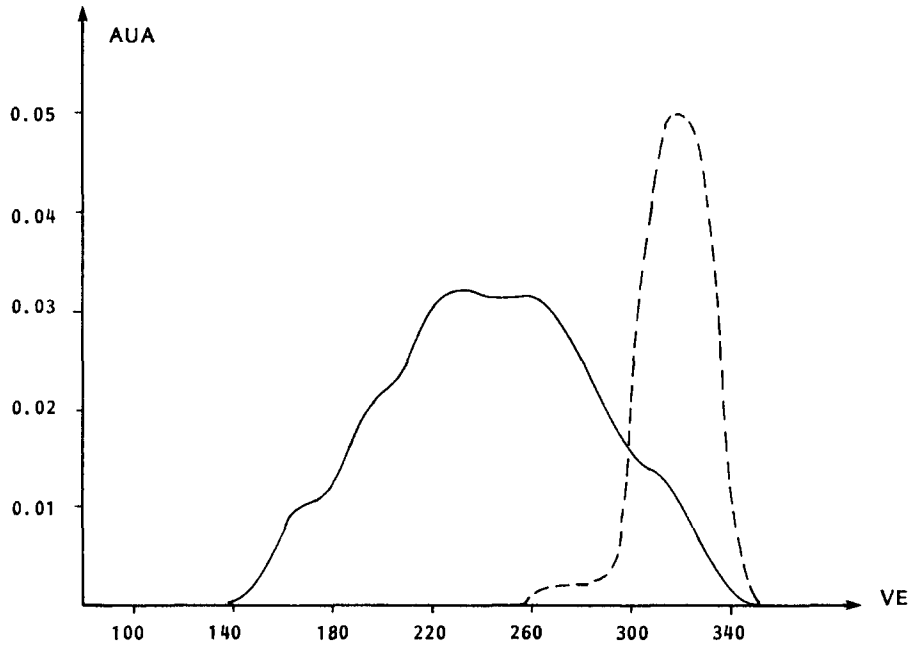

Fig. 1. Gel filtration of a pectin before (——) and after (– – –) degradation by pectin lyase (AUA, anhydro-uronic acids (mg ml^{-1}); VE, elution volume (ml)). Conditions as described in the text. The pectin used was GENU Pectin type BB.

Lee (1985) found a difference between pericarp and gelpectin: the pericarp pectin is subject to pectinesterase action during ripening.

HPLC-chromatograms of pectin lyase degraded pectins are shown in Fig. 2(a). Three peaks can always be observed; these three peaks correspond to oligouronic acids with 0, 1 and 2 non-esterified residues (de Vries *et al.*, 1983). This can be confirmed by using partially esterified oligouronic acids: the three peaks are always observed irrespective of the degree of esterification. Fragments with two to five acid galacturonate residues are only present in small quantities. Fragments with more than five acid galacturonate residues cannot be observed by our method; it can therefore not be concluded yet that 'blockwise' distributed galacturonate residues are absent. But our results are inconsistent with a distribution of the methoxyl groups which is based upon 'block'-sequences. Figure 2(b) shows the distribution of oligomers after cold de-esterification of the degraded pectins;

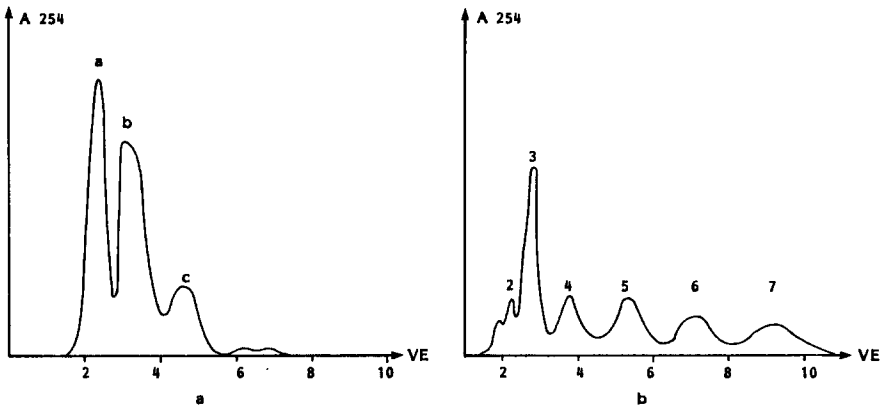

Fig. 2. High-pressure liquid chromatograms of pectin lyase degraded citrus pectins before (a) and after (b) de-esterification (A_{254} = absorbance at 254 nm). HPLC conditions are described in the text.

oligomers with a degree of polymerization of more than 7 cannot be observed by our method.

'Native' pectins with a high DE can be degraded to a greater extent than transesterified pectins (Fig. 3). This clearly illustrates that there is a difference in distribution of methoxyl groups between the two. A very sharp increase in degradability can be observed between DE = 65 and 75 for native pectins. This sharp increase can be explained by assuming the presence of two or more types of pectin with different degradability. Several models can be constructed that are consistent with the curve in Fig. 3. One possibility is to assume that pectin is synthesized with a regular distribution of methoxyl groups, but that the molecules are subject to some random de-esterification after synthesis. A pectin constructed of (MeGalA-MeGalA-MeGalA-MeGalA-GalA)$_n$ has a DE = 80% and can be degraded to 30% if one assumes that the enzyme needs three or more adjacent ester groups for its action. Pectins from lemon, lime, orange and apple seem to fit in the same curve, although it must be said that the authors investigated only one apple pectin sample.

Figure 4 shows the ratios of the peaks a, b and c from Fig. 2(a). As expected, the ratio a/b increases with DE in the case of transesterified pectins: the higher the DE, the more completely esterified are the fragments. The ratio c/b shows for transesterified pectins a somewhat

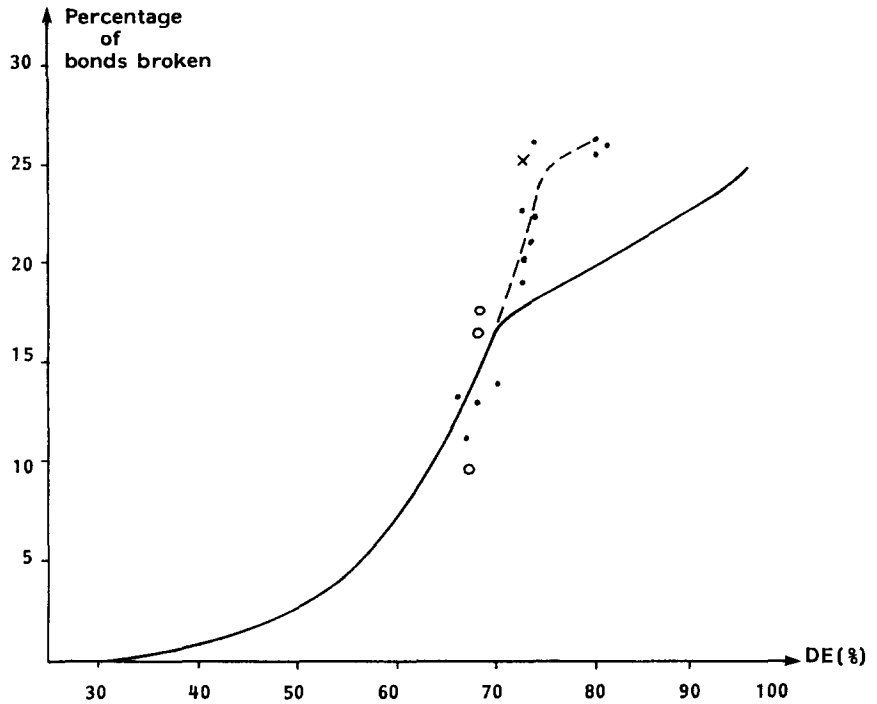

Fig. 3. Extent of degradation of pectins degraded by pectin lyase (——, transesterified pectins (see 'Materials' section); – – –, unmodified pectins; ●, lemon and/or lime pectin; ○, orange pectin; ×, apple pectin). Enzyme degradation as decribed in the text.

more complex curve; also in Fig. 3 and in Fig. 4(a) a 'discontinuity' can be observed at DE 50–60. A possible explanation is that two enzyme substrate complexes are active; alternatively, the enzyme preparation may contain different iso-enzymes.

Figure 4, too, indicates a difference between transesterified and native pectins. For native pectins, the ratios a/b and c/b are independent of the DE. Quite some variation exists, but there is no relation between DE and these ratios. The ratio a/b and also the ratio c/b are higher for transesterified pectins. This implies that in native pectins the amount of isolated non-esterified galacturonate residues is higher, and this points to the presence of some regularity.

Figure 5 shows the distribution of DP in the (de-esterified) degraded pectins. Again, a clear difference between natural pectins

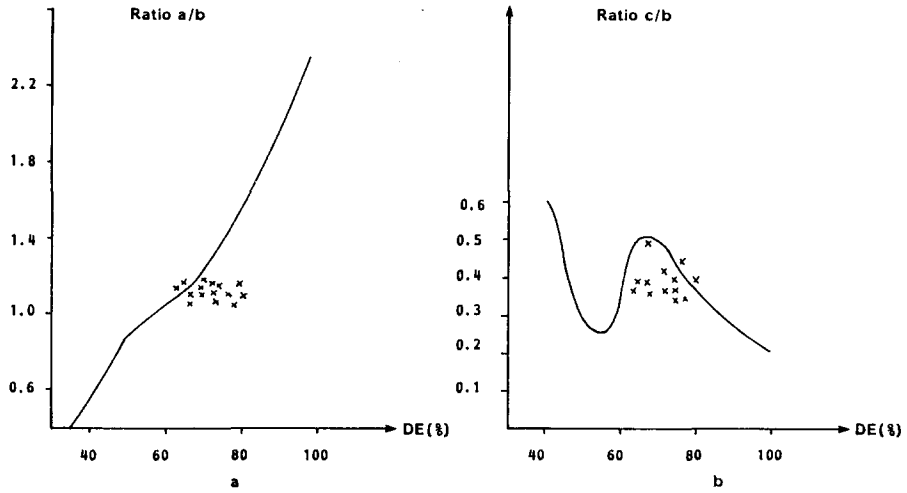

Fig. 4. Ratio peak a/peak b and peak c/peak b (see Fig. 2(a)) plotted against the degree of esterification (——, transesterified pectins (see 'Materials' section); ×, unmodified pectins).

and transesterified pectins is obvious. The high amount of trimers results from the fact that the enzyme cannot degrade trimers (Voragen, 1972): they tend to accumulate.

A striking feature is that the oligomer-patterns in both cases are constant and independent of DE and source of pectin. The patterns are always as shown in Fig. 5, only the total amounts vary with DE (Fig. 6). This is unexpected, e.g. a transesterified pectin degraded to 10% is expected to show a Gaussian distribution of DP around DP = 10. This cannot be explained by assuming a high 'degree of multiple attack', because the pectins were extensively degraded. The explanation may be that the binding enzyme–substrate involves a relatively high number of residues: perhaps eight (van Houdenhoven, 1975). Probably only octamers with a high DE can act as productive binding regions. Another factor may be that the enzyme has an optimum DP as in the case of some polygalacturonases (Dongowski *et al.*, 1983; Dongowski & Bock, 1984). Simulating enzyme attack by the use of computers with randomizing programs as applied by McCleary *et al.* (1984) may yield valuable information.

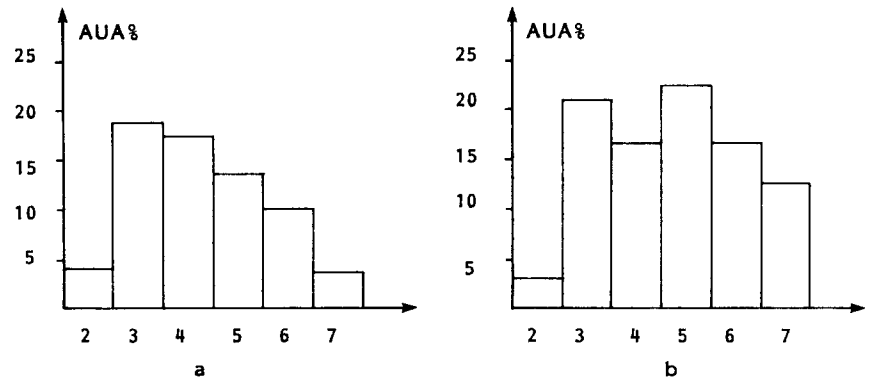

Fig. 5. Distribution of the degree of polymerization (DP) in transesterified pectins (a) and unmodified pectins (b) degraded by pectin lyase. The pectin was GENU Pectin type BB; the transesterified pectin (see 'Materials' section) had a degree of esterification of 70%. The distribution of DP was determined by high-pressure liquid chromatography of de-esterified pectin lyase degraded pectins. Conditions of enzymatic attack and of HPLC were as described in the text.

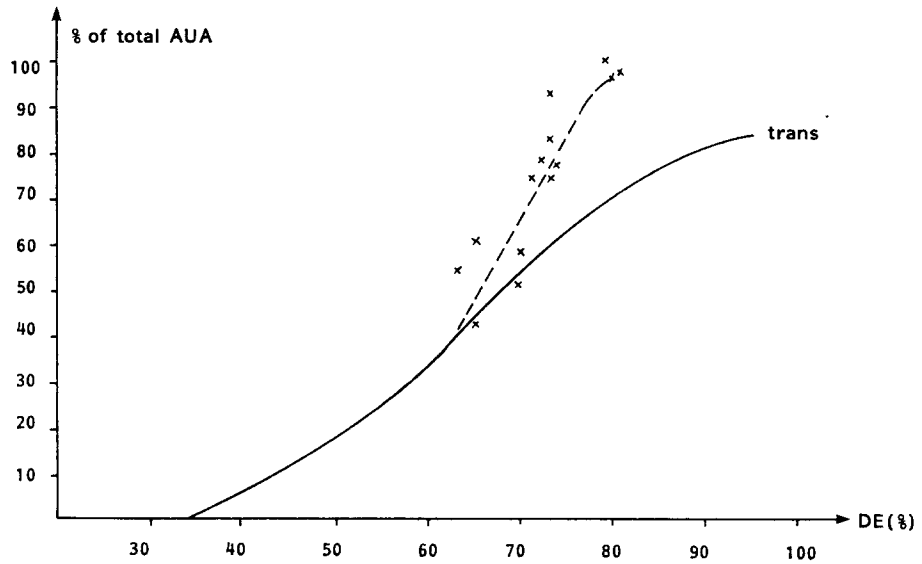

Fig. 6. Percentage of oligouronic acids with a degree of polymerization between 2 and 7 in pectin lyase degraded pectins plotted against the degree of esterification (—, transesterified pectins; ×, unmodified pectins).

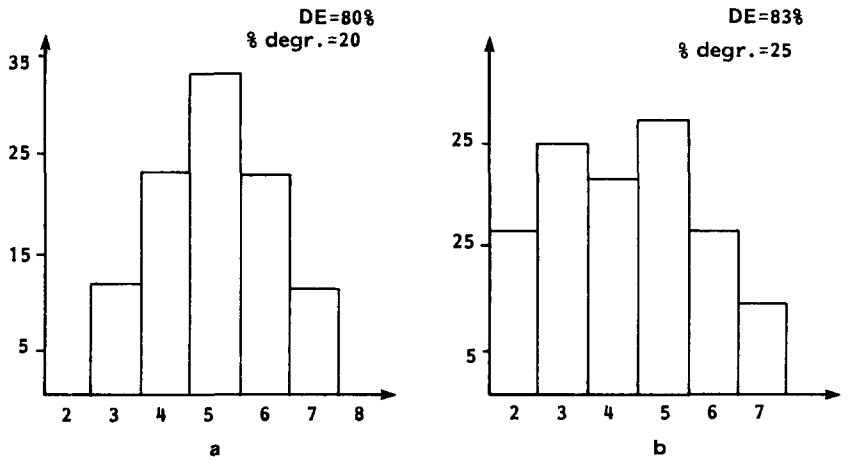

Fig. 7. Theoretical distributions of the degree of polymerization (DP) in pectins of completely regular distribution of methoxyl groups after complete degradation by pectin lyase: a distribution, $[(MeGalA)_4GalA]_n$; b distribution, $[(MeGalA)_5GalA]_n$. It is assumed that the enzyme can only attack sequences of four esterified residues or more.

Whatever the explanation may be, it is clear that there is a difference between native and transesterified pectins. It can be seen in Fig. 5 that in the case of unmodified pectins a relatively high amount of pentamers is present. As we also observed a high amount of isolated non-esterified residues this pentamer might be well represented by MeGalA-MeGalA-GalA-MeGalA-MeGalA. An interesting fact with respect to the mode of action of the enzyme is that the methoxyl groups can be replaced by amide groups without much effect on the enzyme action (results not shown).

Our results show that in native pectins a higher proportion of degradable bonds is present than in transesterified pectins.Possibilities are $[(MeGalA)_4GalA]_n$ or $[(MeGalA)_5GalA]_n$. Figure 7 shows some theoretical degradation patterns. The pattern obtained for $[(MeGalA)_5GalA]_n$ is quite similar to the ones observed, although it does not account for the presence of peak c in Fig. 2(a).

CONCLUSION

This study leads to the conclusion that the distribution of methoxyl groups in pectins is not a random one, not a completely (short range)

regular one, and that the distribution is not based on blocks of esterified and non-esterified galacturonate residues. A positive conclusion is that a relatively high amount of isolated non-esterified residues is present. It may very well be that pectins are synthesized as $[(MeGalA)_5GalA]_n$, but soon lose some methoxyl groups due to chemical and enzymatical de-esterification. In the plant cells, pectin is synthesized as polygalacturonic acid, which is subsequently esterified (Kauss, 1974). A crude enzyme preparation from mung beans can catalyse the esterification of pectate using *S*-adenosyl-methionine as methyl donor (Woodard *et al.*, 1981). It would be interesting to study this enzyme system and to determine the DE of the resulting pectin.

An alternative is that the pectin is synthesized with a DE = 100%, but that the plant pectinesterase reduces the DE to 70%. As stated in the introduction, it is generally assumed that pectinesterase action leads to a blockwise distribution, but it is possible that the enzyme only acts on one side of the helix and de-esterifies, e.g., every third residue. Such a distribution is perhaps very Ca-sensitive. Experiments with pectinesterases of plant origin have been performed at pH 7, but the enzyme may act differently at pH 4. The K_m value of orange pectinesterase increases with decreasing pH (Versteeg, 1979). Our future research will involve the enzymes pectate lyase, polygalacturonase and pectinesterase. We hope to determine the amount of blocks of non-esterified residues both in native pectins and in pectinesterase treated pectins. Also possible differences between apple and citrus pectins will be investigated.

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