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INTRAMOLECULAR DISTRIBUTION OF CARBOXYL GROUPS IN LOW METHOXYL PECTINS – A REVIEW

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ABSTRACT

Low methoxyl pectins (LMP) have been manufactured since the 1940s primarily for use as gelling agents. At the time, it was noted that low methoxyl pectates (LMPs) prepared by different methods had different gelling properties and this was attributed to the way in which the free carboxyl groups were distributed along the chain following deesterification. Various workers have shown that LMP prepared by enzymic deesterification is more heterogeneous with respect to the degree of esterification (DE) than LMPs of the same DE prepared by acid deesterification. This, together with enzyme studies, has been taken as a sign that pectinesterase works in a sequential fashion and, similarly, acid deesterification is a random process.

More recently, the preparation of crude enzyme-deesterified LMP, which is used as a thickener in canned goods, has been described. Although enzyme-deesterified LMPs appear to form weak gels with calcium ions at room temperature (acid-deesterified LMP/calcium gels are reported to be stronger) they are superior when incorporated into canned goods which receive a severe heat treatment.

This review briefly describes the preparation of LMP and that work on LMP which provides information about the distribution of carboxyl groups in the pectate molecule. Since it is established that this distribution affects the gelling properties of the pectin a sound understanding of the chemical aspects may assist in understanding the mechanism of gelation.

METHODS OF DEESTERIFICATION AND THE PRODUCTS

Four methods of deesterification exist, namely, by means of acid, alkali, enzyme and ammonia.

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Acid Deesterification

Treatment of pectin with acid (Woodmansee & Baker, 1949) at low temperatures leads to substantial deesterification with little chain breakdown. However, under extreme conditions decarboxylation may occur (Stutz & Deuel, 1958) as well as chain breakdown. Besides deesterification, acid treatment also removes 'ballast', i.e. sugars other than galacturonic acid (Speiser *et al.*, 1947). An extracted native pectin contains typically 70–80% galacturonic acid which after acid treatment rises to 95% and above (Speiser *et al.*, 1947). This is of importance when calculating DE values from papers which give just percentage methoxyl values. In this review, such adjustments have been made.

The deesterification by acid is considered to be totally random (Speiser *et al.*, 1947) and the resulting LMP is assumed to have the remaining methoxyl groups distributed in a random manner. However, this depends to some extent on the original DE of the pectin. Native pectin is often quoted as having a DE of around 70% and while this is true of the common apple and orange sources, other materials may have a substantially lower DE (Speirs, 1979). Clearly, if the initial material has a low DE, then the final distribution will depend on the initial distribution as well as the method of deesterification employed.

One of the disadvantages of acid deesterification is the long reaction time required to produce a suitable low DE, for example, in dilute acid at 50°C a 1·3% pectin solution of initial DE 80% requires 50–100 h hydrolysis to give a DE of 10% (Speiser *et al.*, 1945). Shorter hydrolysis times (4 h) can be achieved but only with considerable depolymerisation (Woodmansee & Baker, 1949). This disadvantage coupled with the need for acid resistant equipment has led to a closer examination of the other deesterification methods.

Alkaline Deesterification

The use of alkali is the classical chemical method of splitting ester links, but in the case of pectin, deesterification is accompanied by depolymerisation of the pectin chain. Vollmert (1950) noted that depolymerisation was related to the methoxyl content of the pectin and that pectic acid was resistant to alkaline depolymerisation. In 1958 Neukom & Deuel suggested that degradation occurred by β-elimination. However, despite the problem of depolymerisation, careful control of the process can produce an acceptable LMP (McCready *et al.*, 1944; Owens *et al.*, 1949).

The rate of alkaline deesterification is more rapid than in the case of acid deesterification and reaction times of the order of 1–2 h are sufficient to lower the DE from 80% to under 10% (e.g. Vollmert, 1950).

Recently some doubts have been expressed about the randomness of alkaline-deesterified LMP. Fielding (1975) in his review of LMP production wrote 'Partial hydrolysis of the ester groups by alkali may give the third type of distribution pattern, i.e. an even, regular distribution'. No evidence is presented that fully supports this suggestion although some minor differences in the carboxyl distribution of acid and

alkali deesterified LMP have been detected by chromatographic methods (Heri *et al.*, 1961). These are discussed later.

Enzymic Deesterification

The attractions of enzymic deesterification for the industrial preparation of LMP are manifold. First, enzymic deesterification is rapid. Second, the pectinesterase enzyme which causes deesterification is normally found in association with pectin in plant material and under suitable conditions deesterification may be accomplished *in vivo* (see for instance, California Fruit Growers Exchange, 1937; Schultz *et al.*, 1945; Mitchell *et al.*, 1978a; Speirs *et al.*, 1980; Taylor & Pritchard, 1981). Alternatively pectin may be first extracted and then treated with pectinesterase. Orange pectinesterase is most frequently used, as orange peel contains no polygalacturonase (MacDonnell *et al.*, 1945) and thus crude enzyme preparations can be used with no danger of depolymerisation. When the natural pectinesterase of a plant material is utilised for the *in situ* deesterification, the material is normally minced to release the pectinesterase and the pH of the macerate is made alkaline to favour the pectinesterase while disadvantaging any polygalacturonase which has an acid pH optimum. Care must be taken in adjusting and maintaining the pH otherwise inadvertent alkaline deesterification may take place. Normally it is considered that deesterification below pH 8·5 is enzymic and above 8·5 alkaline deesterification becomes significant. The deesterification may be speeded by raising the temperature to around 50–60°C (Taylor & Pritchard, 1981). Other work has suggested that in intact tissue a 'heat shock' increases the activity of extracted pectinesterase (Taylor *et al.*, 1981). This may also prove beneficial in producing LMP.

It is generally considered that enzymic deesterification is a sequential process. Many workers have presented evidence to support this observation. The work which describes the distribution of carboxyl groups in LMP will be discussed in the next section. Possibly the most elegant and conclusive proof of the actual site of pectinesterase attack was provided by Lee & MacMillan (1970). These workers showed that tomato pectinesterase, when confronted with an almost fully esterified pectin, attacked at the free reducing end of the chain or at one of the few free carboxyl groups. Thus the distribution of free carboxyl groups on the native pectin molecule affects the rate of attack by pectinesterase. Solms & Deuel (1955) showed that when pectins of 50% DE prepared by alkaline and enzyme treatment of fully methylated pectin were reacted with pectinesterase, the pectin prepared by alkali treatment was deesterified more rapidly. This observation was explained by a different distribution of free carboxyl groups.

In comparison with the enzyme-deesterified pectin, the alkali-deesterified pectin had many more sites where a free carboxyl was adjacent to a methoxyl and at which pectinesterase could attack. The practice of adjusting the operating pH of pectinesterase to alkaline to 'increase the activity' may therefore have more to do with limited deesterification of the pectin by the alkali providing more sites for attack by

the enzyme than the classical pH/conformational changes normally associated with optimal enzyme activity.

Ammonia Deesterification

The reaction of pectins with ammonia in alcohol or in aqueous ammonia produces LMP which have some degree of amidation (Joseph *et al.*, 1949; Bryant 1959; Wiles & Smit, 1971; Black & Smit, 1972b). What role these amide groups play in gelation is not known. Little else is known about their properties and no information on the distribution of the carboxyl groups is available.

DISTRIBUTION OF CARBOXYL GROUPS IN LMP AND GELLING STRENGTH

Several methods have been used to determine the distribution of carboxyl groups in LMP samples prepared by acid, alkali and enzyme deesterification. The information tends to be qualitative and has been used to explain the difference in gelling properties between LMPs produced by the different processes. Although the distribution of carboxyl groups plays some role in determining gel strengths, the effect of other factors has not been investigated as thoroughly as one would like.

Early experiments on comparative gel strength all refer to the work of Hills *et al.* (1942). These workers studied the possibility of replacing the conventional 65% sugar high methoxy pectin (HMP) gels found in jams with a 35% sugar-calcium LMP gel because, at the time, there was a shortage of sugar due to the War. Their results have been quoted (Speiser *et al.*, 1947) and their method of gel formation used by other workers (Speiser & Eddy, 1946; Speiser *et al.*, 1947; Black & Smit, 1972a; Padival *et al.*, 1979). Hills *et al.* (1942) showed that acid-deesterified LMP formed much stronger gels with calcium than did enzyme-deesterified LMP. However, a reappraisal of their experimental methods throws some doubt on the validity of the above observation. For instance, the enzyme deesterification was performed with an extract from ripe tomatoes which is now known to contain polygalacturonase. Although mention is made of the fact that not all sources of pectase are satisfactory because degradation and loss of gelling power are sometimes observed, partial depolymerisation of pectate may have taken place. This would of course reduce the molecular weight and produce weaker gels in the case of the enzyme-prepared LMP.

The second point about the comparisons is the manner in which the calcium pectate gels were formed. More recent work has shown that with a slow release of calcium, either by dialysis (Smidsrød & Haug, 1972) or by means of a calcium phosphate/glucono-δ-lactone system (Mitchell & Blanshard, 1976), strong pectate gels can be formed. Unlike the gels formed by the method of Hills *et al.* (1942) which showed an optimum calcium/pectinate ratio, the gels formed by a slow calcium release obtain a plateau value with excess calcium.

Third, the currently favoured model of calcium pectate gels (the junction zone egg box model originally proposed by Grant *et al.*, 1973) envisages the interconnection of

pectate molecules through calcium linkages between adjacent regions of 15 to 20 galacturonic acid molecules (Kohn & Luknor, 1977).

If this is valid, then for LMP preparations above 5% DE, enzymically deesterified LMP should contain more such regions than the randomly deesterified acid-prepared LMP. Assuming other factors (e.g. molecular weight) are equal then at DE values above 5% it would be expected that enzyme-deesterified LMP calcium gels would be stronger. This is not reflected in the results of Hills *et al.* (1942).

Unfortunately, no one has compared and recorded the strengths of calcium LMP gels which contain LMP prepared by acid and enzymic means (matched with respect to DE, molecular weight and galacturonic acid content) and which have been gelled with a slow release of calcium. This is an area long overdue for investigation.

Although attention has been focused on gel production in jams and desserts, there appears to be an application in canned goods (Mitchell *et al.*, 1978b). By using a system which releases calcium ions on heating, the canned pack can be thickened or gelled in the late stage of processing, thus effecting high rates of heat transfer. For this process to be effective, a LMP with extremely low DE (<20%) is required, otherwise extensive depolymerisation will occur because of β-elimination (Speirs *et al.*, 1980).

METHODS FOR DETERMINING CARBOXYL DISTRIBUTION IN DEESTERIFIED PECTINS

In chronological order, the methods used to study the distribution of carboxyl groups in LMP are presented below.

Speiser *et al.* (1947) studied the electrophoretic mobility of LMP samples of similar DE prepared by acid (32% DE) and enzyme (35% DE) deesterification of the same apple pectin. The differences between the electrophoresis patterns are slight but suggest that the enzymically prepared LMP is more heterogeneous than the acid-prepared LMP. The experiment therefore supports the theory of sequential enzyme deesterification which produces molecules some of which have been totally deesterified and others which have been hardly attacked. The acid-prepared LMP on the other hand has, by the random attack hypothesis, a much narrower range of DEs among the molecules.

Mention should at this stage be made of work by Owens [personal communication cited in Speiser *et al.* (1947)] in which he is reported to have made similar observations on enzyme deesterified citrus pectin which was fractionated by 'chemical means'. The fractions showed a wide range of DE values.

Heri *et al.* (1961) prepared pectinate samples (DE around 50%) by the action of acid, alkali and enzyme on an almost fully esterified (97% DE) pectin. The pectinate samples were then fractionated on a column of DEAE-cellulose. Five distinct fractions were identified and the proportion of sample in each fraction was determined together with the DE of that particular fraction. Table 1 shows the results.

It can be seen that the vast majority of the acid treated sample (89%) is present in fractions II and III and the DEs of these fractions are 54% and 52% respectively. The

TABLE 1
Fractionation of partially deesterified pectins on DEAE-cellulose (Heri *et al.*, 1961, reproduced with permission)

Method of preparation and DE (%)		*Distribution of galacturonic acid in the fractions (%)*					*DE of fractions (%)*				
		I	II	III	IV	V	I	II	III	IV	V
Acid	55	6	62	27	1	4	74	54	52	–	22
Alkali	48	2	49	41	1	7	–	52	45	–	13
Enzyme	56	24	7	30	3	36	92	87	54	37	24

DE of the whole unfractionated sample was 55% so this sample is extremely homogeneous with respect to DE. The alkali treated sample shows some minor differences but the enzyme treated sample is markedly different. Approximately equal amounts are found in fractions I, III and V which have DEs ranging from 92% through 54% to 24%, reflecting the heterogeneity of this sample.

The evidence provided by Heri *et al.* (1961) proves conclusively the hypothesis which has been proposed by many workers and which was supported by much circumstantial evidence, but which had not been directly proven previously.

In 1968, studies on highly esterified pectin (95%) which had been partly deesterified (45-79%) by tomato pectinesterase and potassium hydroxide once again indicated that enzymic deesterification gave rise to blocks of free carboxyl groups (Kohn *et al.*, 1968). In this case, the stability constants of the calcium pectinates were determined and the high stability constant of the enzyme-prepared pectinate was attributed to a higher density of electric charge which in turn was produced by the blockwise enzymic deesterification. Despite attempts to purify tomato pectinesterase some depolymerisation did occur and one glycosidic bond was split for every 80–120 ester groups hydrolysed. This is reflected by the molecular weight of the pectinates which decrease with falling DE. It is interesting to note that this purified tomato preparation caused depolymerisation whereas Hills *et al.* (1942) used a crude extract.

A study on the distribution of free acid groups in pectinic acids by Henfrey (1973) used electrophoresis to separate the products of enzymic deesterification. He deesterified pectin (initially of DE 70%) with orange pectinesterase and noted the heterogeneous nature of the product as had Speiser *et al.* (1947). Further experiments with a highly esterified pectin substrate (DE 95%) and varying amounts of enzyme in which the products were analysed at various times, gave an insight into the manner in which enzymic deesterification takes place. At low enzyme/substrate ratios, the enzyme shows an initial preference for highly methoxylated pectin. The first reaction is a partial deesterification which leads to the accumulation of compounds with electrophoretic mobility intermediate between that of polygalacturonic acid and fully methylated pectin. After the fully methoxylated chains have been converted to this

intermediate stage, a second reaction sets in in which the intermediate material is further attacked to give pectic acids. With high enzyme/substrate ratios these two reactions take place simultaneously and pectic acids are detected immediately on the electrophoretograms.

Henfrey (1973) attempted to further characterise the products of acid, alkali and enzyme deesterification by chemical transelimination, study of the acid dissociation curves and dye binding experiments.

Chemical transelimination could possibly give the exact distribution of carboxyl groups in a pectin sample as the reaction will split the galacturonic acid backbone adjacent to a methoxylated group (Fig. 1). Unfortunately, in practice, deesterification proceeds more rapidly than transelimination which leads to only partial depolymerisation at the methoxylated residues.

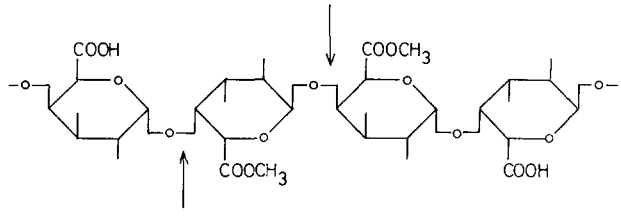

Fig. 1. Potential sites for cleavage of the pectinate chain by transelimination.

Studies on the acid dissociation curves of LMP prepared by acid and enzymic deesterification showed differences which reflect the different carboxyl distributions. The theoretical acid dissociation curve for a randomly deesterified LMP fits the experimental data from the acid deesterified LMP closely. For blockwise deesterification a steeper acid dissociation curve is expected and the enzyme-deesterified material follows the trend except for an inflection at 30–40% neutralisation values. Henfrey suggests that this inflection may be explained by a conformational change in the molecule. This work supports previous observations that acid deesterification is random and enzyme deesterification is blockwise.

Another approach adopted by Henfrey was to use metachromic dye binding studies. The dyes used, acridine orange and methylene blue, bind to free carboxyl groups which show a degree of adjacency. If the amount of dye binding is plotted against the DE of acid and enzyme deesterified samples, it can be seen that the enzyme deesterified material binds more dye in the initial states of deesterification than the acid deesterified samples. This again suggests that enzyme deesterification produces blocks of adjacent carboxyl groups.

Another study by Fielding (1975) looked at methods which might characterise LMP. Following a method proposed by Kotchetkov *et al.* (1970), he attempted to convert the esterified groups on the LMP molecules to other functional groups which

would allow selective breakdown of the galacturonorhamnan chain. The conversion to amido and hydroxamic acid derivatives was attempted but in the former case only 88·2% amidation was achieved with some depolymerisation and in the latter case the reaction also failed to produce a 100% conversion.

The use of pectic enzymes to selectively degrade the LMP molecule was discussed by Fielding (1975) but he pointed out that hydrolysis was not always complete and that oligomers were also only partially degraded.

Recent work in this Department has been directed to the development of a method which might provide quantitative information on the number of adjacent carboxyl groups in LMP (Tuerena *et al.*, 1981). The method is an amalgam of chemical and enzyme techniques and consists of the following steps:

1. The free carboxyl groups on the LMP are 'blocked' with glycol ester.
2. The 'blocked pectin' is reacted with a mixture of pectic enzymes which removes all unblocked residues leaving the 'blocked' residues intact.
3. The 'blocked oligomers' are separated from the other products of enzymic hydrolysis.
4. The 'blocked oligomers' are analysed to determine their molecular weight distribution.

The chosen blocking agent, ethylene oxide, reacts almost totally (90%+) with the free carboxyl groups (Deuel, 1947) and the resulting glycol esters are impervious to attack by the pectic enzymes utilised to effect stage 2. This leads to substantial hydrolysis of the methylated portion of the chain without hydrolysis of the glycolated portion. The subsequent separation and characterisation of the hydrolysis products leads to an estimate of the number average molecular weight of the portions of the chain that originally were free carboxyl groups. Currently, experiments are in progress to assess the validity of the technique with samples of LMP prepared by the various methods. Ultimately, it may prove possible to correlate the distribution of carboxyl groups with the gelling properties of a particular sample.

Although this paper is not intended to be an exhaustive review of the literature, it is hoped that the key papers and relevant facts have been presented. Despite the amount of work on pectin, there are still many gaps in our knowledge, especially in the field of pectin gels.

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