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ENZYMATIC MODIFICATION OF PECTIN IN Ca-PECTIC GELS

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ABSTRACT

Pectin is one of the major structural cell wall polysaccharide of higher plants. Pectin macromolecules include several regions, rhamnogalacturonan I, rhamnogalacturonan II and homogalacturonan, differing in their sugar composition and structure. The main sugar is D-galacturonic acid, which can be methyl-esterified. The degree of methylation (DM-defined as number of methylesterified galacturonic acids per 100) is an important parameter related to mechanism of gelation.

Enzymatic demethylation influences the gelation of pectins. Pectinmethylesterases (PME) from plant and fungal origin have different mode of action – blockwise and random respectively and produce pectins with different gelling properties. Hence for correct description of the gelling pattern of pectins, not only DM but also the distribution of free carboxylic groups is to be known. Degree of blockiness (DB) is a quantitative measurement of the blockwise distribution of free carboxylic groups in pectin molecule.

In the present work pectins with different DM were obtained as a result of demethylation with PME from orange and *Aspergillus aculeatus* in 50mM MES buffer at pH 6, 30°C and presence of 3mM $CaCl_2$. Their degree of blockiness was determined and was showed the ability of HM pectin demethylated with orange PME to form a gel in the presence of Ca ions even at DM=70. In any case pectin demethylated with fungal PME doesn't gel if DM>50. The changes of DM and DB were followed along the demethylation process and were related to the gelling properties of pectin monitored by rheological measurements in oscillatory shear.

Keywords: pectin, PME, enzymatic modification, pectin gelation

INTRODUCTION

Pectin is a natural complex biopolymer found in primary plant cell walls which backbone is built mainly by galacturonic acid [1]. Despite its structural complexity and diversity, three major building subunits are generally recognized – homogalacturonan, rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). Homogalacturonan consists of a long linear chain of α-(1→4)-galacturonic acid (GalA). RG I has a backbone built by repeating disaccharide unit [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→] bearing neutral sugars side-chains (arabinan, (arabino)galactan) linked to the rhamnose residues. RG II has a backbone similar to homogalacturonan with attached very complex side chains. The carboxyl groups of the GalA units are usually methylesterified and the molar ratio of methoxyl groups per 100 GalA is defined as the degree of methoxylation (DM). Thus two major types could be defined – Low Methoxy (LM) pectin when DM is lower than 50 and High Methoxy (HM) pectin when the DM is higher than 50. The main application of pectin is as gelling agent in food industry.

The presence of free carboxyl groups or the possibility to deesterify the metoxylated carboxylic groups gives the pectin polyelectrolyte behaviour. One of its main characteristic is certainly its capacity to bind calcium ions and to be cross-linked through these ions. Such calcium-mediated structures have been claimed to be present in the cell walls, leading to insolubilisation of pectins: this is one of the mechanisms proposed for the linkages between polysaccharides in the cell walls. The extraction of pectins by calcium chelating agents [2] as well as the recognition of such structures by monoclonal antibodies [3] are often presented as proofs for this hypothesis, although the pectins present in these extracts are generally highly methylated and sometimes acetylated, ruling out the probability of long free GalA sequences [4].

DM is an important parameter influencing the process and mechanism of association of pectins but the distribution of the unesterified GalA residues in the pectin macromolecule is more and more recognized as having a critical role in these associations since it affects the reactivity of pectin with cations. Usually HM pectins gel at low pH and in presence of sugars and LM pectins gel in presence of divalent cations. Indeed blockwise distribution of free carboxylic groups favors the calcium binding much more than a random one. The use of chemicals and enzymes for deesterification may lead to different esterification patterns. When studied in solution, it is well known that acidic deesterification or fungal pectin methylesterases (PME, E.C. 3.1.1.11) cause random distribution of the free GalA whereas plant PMEs are known to lead to blocky structure [5-9]. A new parameter – degree of blockiness (DB) [10], was introduced as an attempt of quantitave measurement and characteristic of distribution pattern of free carboxylic groups, trying to better describe relationship structure-gel-forming properties of pectins. DB measurement is based on the determination (by HPAEC [10], capillary electrophoresis [11], NMR-spectroscopy [5, 6]) of the products (non methylated monomer-1°, dimer-2° and trimer-3° of GalA) of extensive enzymatic digestion of pectins by endo-

polygalacturonase, which prefers substrate with long unesterified blocks of GalA (eq.1) [10].

$$DB = \frac{[(1\times 1°)+(2\times 2°)+(3\times 3°)]M_w^{GalA}}{(1-DM/100)m_{pectin}(m_{uronicacid}/m_{pectin})}\times 100 \quad (1)$$

The more blocky structure has the pectin the greater will be the quantities of liberated monomer, dimer and trimer of GalA and thus the degree of blockiness will have higher values compared to pectins with a random pattern of deesterification.

The aim of the present work is to investigate the process of enzymatic demethylation using PMEs from orange and *Aspergillus aculeatus* in an *in situ* mediated Ca-pectic gel. The changes of DM, DB, G' and G'' (storage and loss moduli) were monitored and used to characterized the gel system.

MATERIALS AND METHODS

The lime pectin used was kindly provided by Cargill Texturizing Solution (Redon, France). P71 is a HM pectin with DM=71 (containing 81.8% GalA).

Pectin methylesterases used were from orange (*O*-PME, Sigma P5400, L'Isle d'Abbeau, France) and from *Aspergillus aculeatus* (*Aa*-PME, UniProt Q12535), the later one being kindly provided by Novozymes A/S (Copenhagen, Denmark). The enzymes were solubilized at 5mg/ml in 2-[N-Morpholino] Ethane-Sulphonic acid (MES) buffer (10 mM, pH 6) and dialyzed overnight at 4°C against the same buffer. Before enzymatic incubation the enzymes were diluted as necessary with the same buffer.

The endo-polygalacturonase (AnPGII, E.C. 3.2.1.15, UniProt P26214, provided by Novozymes) was from *Aspergillus niger*.

The GalA content of the pectins was colorimetrically determined by the automated m-hydroxybiphenil method [11].

DM of the pectins deesterified by PMEs were determined by quantification of values of methanol released by alkaline deesterification (0.5 M NaOH) for 1h at 4°C in presence of $CuSO_4$. For their determination reverse phase HPLC was carried out with a C18 Superspher column (Merck) using 4 mM H_2SO_4 as solvent at a flow rate of 0.7 ml/min at 25°C. Isopropanol was used as internal standard and DM was calculated as the molar ratio of methanol to GalA.

Rheological measurements were performed at 30°C using controlled-stress rheometer (AR2000, TA instruments) equipped with a Peltier temperature controller and with a cone-plane device (20 mm diameter, 4° angle, gap between cone and plane 113 μm). Preheated at 50°C 2% pectin solution and 6 mM $CaCl_2$ (both in 50 mM MES buffer, pH 6) were mixed by adding slowly $CaCl_2$ solution at equal volumes (1% pectin and 3mM Ca final concentration). The enzyme was added at the necessary activity, solution was well mixed and approximately 1.5mL was transferred onto the rheometer plate also preheated at 50°C. The visco-elastic properties of pectin gels

were characterized by measuring the storage (G') and loss moduli (G") over time of gel formation and evolution. Time sweep test was done at a frequency of 1 rad/s and strain amplitude 1% followed by frequency sweep test at the same deformation rate.

RESULTS

Treatment of P71 and gel evolution. Since DM of pectins is an important parameter determining their physico-chemical properties, kinetic of deesterification of P71 was followed for 24h using different enzyme activities. Incubation of pectin with *Aa*-PME led to very fast decrease of DM compared to *O*-PME (Table 1 and 2).

Table 1. *Change of DM of P71 deesterified by Aa-PME for 24 hours*

Time, h	Activity				
	7.11nkat	**3,55nkat**	**1.19nkat**	**0.24nkat**	**0.08nkat**
0	71.2	71.2	71.2	71.2	71.2
1	30.85	48.85	35.4	60.1	61.5
2	27	45.55	29.8	55	60.4
4	21	40.2	29.4	41.7	57.7
6	15.15	31	29.1	32.4	49.5
8	11.75	27.8	26.1	30.4	48.8
10	8.5	29	27.6	30.6	43
24	4.1	9.45	19.6	28.9	28.9

After 24h incubation for all the samples deesterified with *Aa*-PME the DM was below 30. At the highest activity used (7.11 nkat) *Aa*-PME deesterified pectin to very low DM. For the *O*-PME decrease of DM was moderate (Table 2) and at activities of 0.08 and 0.24 nkat DM was still above 50 after 24 hours.

Table 2. *Change of DM of P71 deesterified by O-PME for 24 hours*

Time, h	Activity				
	7.11nkat	**3.55nkat**	**1.19nkat**	**0.24nkat**	**0.08nkat**
0	71.2	71.2	71.2	71.2	71.2
1	67.7	66.1	70.2	69.7	70.6
2	56.7	54.6	67.4	66.9	66.9
4	53.5	49.7	67	63.9	67.1
6	45.1	48.1	66.6	58.5	66.5
8	44.8	43.7	62.4	55.8	65.2
10	43.9	43.1	57.8	55.5	65.1
24	36.5	35.2	55.9	51.7	59.3

Along with the DM decrease system evolution was investigated by time sweep oscillatory measurements and attempt to connect changes of DM with rheological behavior of the gels was made. Although DM of samples treated by *Aa*-PME decreased very fast in the beginning this led to moderate increase of storage and loss moduli (G' and G'' respectively) – Fig.2. For activities of 1.19 and 0.24 nkat gels continued to evolve and equilibrium in the system was not reached after 24 hours.

One explanation for this observation could be the redistribution of Ca ions along the pectin macromolecules with the new free carboxylic groups formed during the constant action of PME. For the lowest activity – 0.08 nkat equilibrium was reached relatively fast but with very low values of G' and G''. Gel evolution for the samples deesterified with *O*-PME was a very rapid process even that the DM was higher than 50 and the higher is the activity of the enzyme used the more pronounced was this effect. Whatever the amount of *O*-PME added in the medium (except the lowest activity) the values of G' at the end of the incubation were higher than those obtained with *Aa*-PME. These observations demonstrate that the most important parameter for the gelation process and gel evolution is not the decrease of DM but rather the pattern of introduction of free carboxylic groups in pectin during the deesterification.

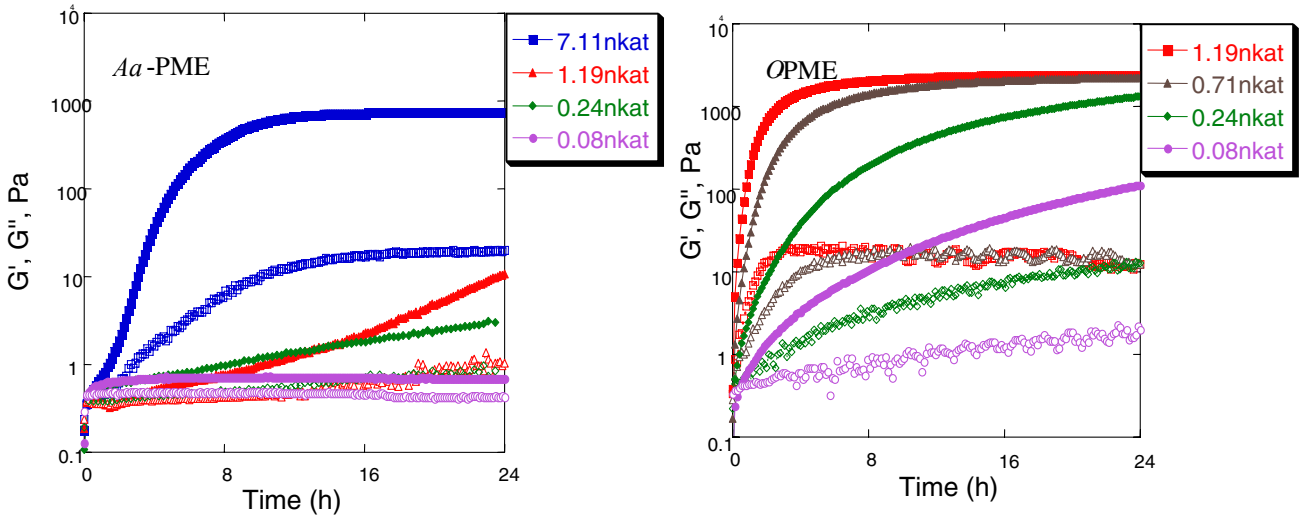

Figure 1. *Storage and loss modulus (G'-filled figures; G''-empty figures) obtained for 1% HM pectin with different activities of A-PME and O-PME*

DM and DB comparison. Confronting data of DM decrease for both PMEs treatment and rheological properties of the pectin gels obtained as a result of PME action has shown that more important parameter for gel ability is the pattern of distribution of free carboxyl groups and not the simple decrease of DM. To obtain information about the pattern of deesterification we performed enzymatic fingerprinting of the deesterified pectins using endo-PG II. Since the preferred substrate of endo-PG II is polygalacturonic acid or pectins having long blocks of free GalA the more blocky structure has the pectin the more products of enzymatic degradation will be obtained.

Even the smallest decrease of DM of pectins treated with *O*-PME resulted in an increase of DB from 10% for the untreated pectin to about 18% (after 120 min of treatment with 0.24 nkat). After 24 hours the DB was higher than 64% even if the DM was above 55. On the contrary for *Aa*-PME the large decrease of DM was accompanied by moderate increase of DB. At the higher activity used (1.19 nkat) after 24 hours DM was below 20 but DB reached values of approximately 30%.

The importance of presence of block structures in pectins can be seen when DM decrease, DB increase and gel evolution are plotted together – Fig. 2. The increase of

DB for samples deesterified with *O*-PME corresponds to the increase of G' of the system and the more steep rise of DB is observed the more sharp is the gel evolution. For the *Aa*-PME moderate increase of DB is observed although the sharp decrease of DM. This resulted in a slowly evolving gel system, which did not reach equilibrium for the time of measurements. The formation of gels using *Aa*-PME occurred when the DM decreases below 20 and in this case presence of more blocky structures is due to the low DM (data not shown). The moment of visual gelation of the *O*-PME deesterifed pectin is after 120 min of the incubation. Gelation strongly depends from DB increase and was done even at the values of DM 67.4. For *Aa*-PME – no visual gelation was observed for 24 hours with this enzyme activity even the final DM was below 30.

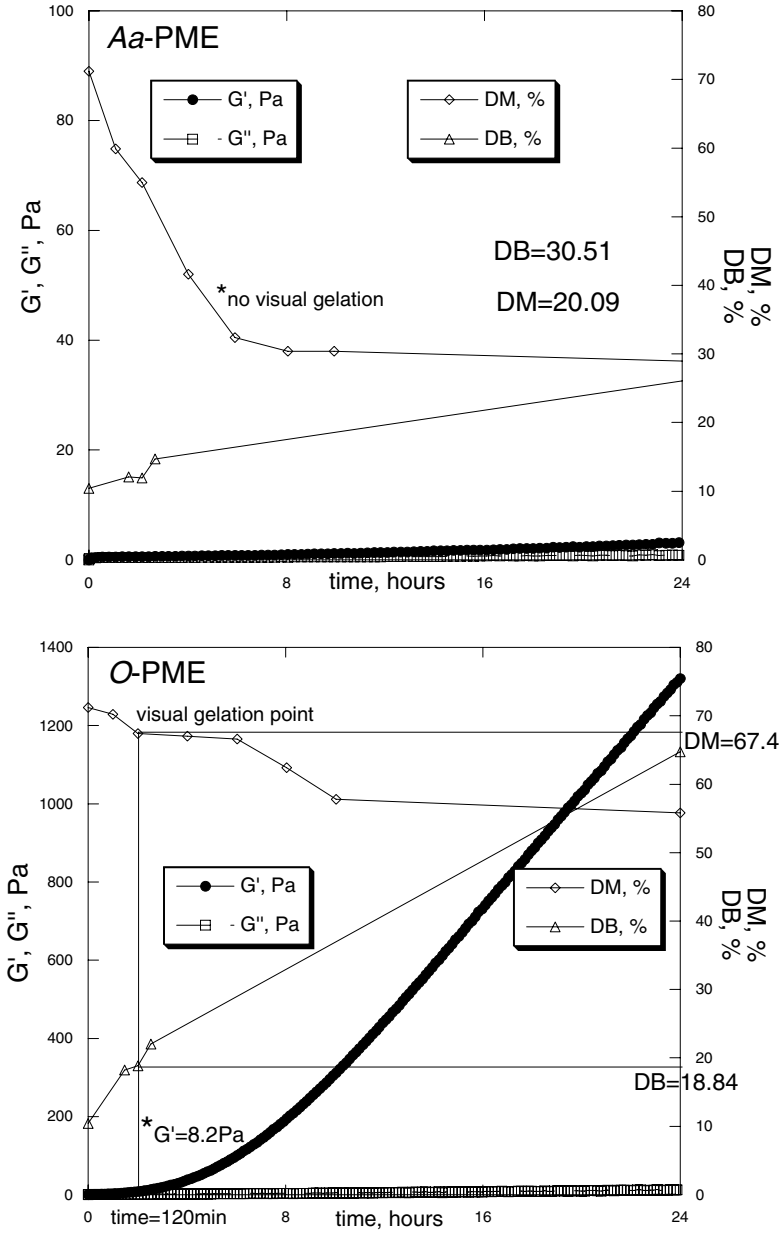

Figure 2. *DM, DB, G' and G'' change for 24h incubation of 1%HM pectin, 3mM Ca, 0.24nkat PME - 1. Aa-PME, 2. O-PME*

CONCLUSIONS

Orange PME-deesterified pectin gives strong gels although the high DM of the pectin. Gelification is faster than by using fungal PME and evolution of the gel system is related to DB. *Aa*-PME treatment of pectins leads to fast demethylation and after 24 hours for all activities used DM is below 50. DB describes better gelation process for both enzymes but is not the only factor which influences it. The presence of PME during the gel formation allows to delay the equilibrium and thus to propose new stimulable gelling systems. Moreover, the enzyme behavior observed in this work gives new insights of the role of calcium in muro.

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