

# Divalent Cation Interactions with Oligogalacturonides

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The conformational properties of high and low molecular weight galacturonides were investigated in relation to the ability of oligomers with degree of polymerization  $>10$  to act as elicitors of plant defense mechanisms. Oligomers from polygalacturonate were obtained by means of enzymatic hydrolysis. Two fractions exhibiting high and low average degrees of polymerization were isolated by solvent fractionation and characterized by means of electrospray mass spectrometry. The conformational behaviors of the two fractions were investigated in the presence of different divalent cations using circular dichroism. Calcium, copper, and zinc ions were able to induce a conformational transition in both fractions. When in the presence of the high molecular weight fraction, copper and zinc ions were much more effective than calcium ions, whereas the efficiency was much reduced with low molecular weight oligomers.

**Keywords:** Galacturonides; conformational properties; cation interaction; electrospray mass spectrometry

## INTRODUCTION

Polygalacturonic acid is a gelling polysaccharide that constitutes the chemically homogeneous fraction of pectins (1, 2). Pectins are located in the extracellular matrix of soft tissues of fruits, and they normally exhibit variable esterification degrees, branching, and other minor chemical inhomogeneities (3–6). Sol–gel transition in polygalacturonate solutions is induced by divalent cations, in particular, calcium ions. The currently accepted model of gel structure involves an extended and interconnected polymeric network composed by both aggregated secondary structure domains and cavities able to retain water and maintain the gel swollen. The schematic conformation of secondary structures is indicated in the literature as “egg-box” arrangement (7, 8).

Pectins and polygalacturonates are extensively used in the food industry. However, besides technological applications, galacturonans are also investigated because they play an important role in plant physiology. In fact, oligogalacturonides produced by exogenic endogalacturonases elicit plant defense mechanisms against pathogenic agents such as fungi (9–12). The conformational features of both poly- and oligogalacturonans are very likely involved in their chemical and biochemical properties. These include interactions with positively charged ions, in particular, divalent cations, which promote secondary structure formation.

In the frame of a research project aimed at the structural investigation of gelling polysaccharides (13–15), the conformational characteristics of oligogalacturonides obtained by means of enzymatic hydrolysis and exhibiting different molecular weight were investigated in the presence of different divalent cations. In fact, it has already been demonstrated that only oligomers with degrees of polymerization (DP)  $>10$  exhibit certain

biological activity, the most active being the dodecamer (9, 16). A study on the conformational behavior of oligogalacturonates carried out by means of circular dichroism (CD) was published 20 years ago (17, 18), but these authors limited their analysis to oligomers with DP from 2 to 5, DP = 44, and DP = 340. Therefore, specific effects connected with fractions having DP  $\sim 10$  were neglected, and only calcium complexes were investigated.

## MATERIALS AND METHODS

**Preparation of Oligogalacturonate Fractions.** Polygalacturonate oligomers were obtained by enzymatic hydrolysis using an endopolygalacturonase produced by the fungus *Fusarium moniliformis* and expressed in modified yeast cells (16, 19, 20). For enzyme production, yeast cells were grown in 200 mL of YPD liquid medium (10 g of yeast extract, 20 g of triptone, and 20 g of galactose for 1 L of distilled water) at 30 °C for 4 days. The enzyme secreted in the cell culture medium was subjected to a partial purification consisting in a dialysis against 20 mM acetate buffer, pH 4.8. The enzyme concentration was determined only indirectly, by measuring the reducing ends (21) produced as a function of time upon reaction with polygalacturonate. In this way, on the basis of the average DP obtained, appropriate conditions (reaction time and enzyme concentration) for the enzymatic degradation were established. Hydrolyses were carried out by adding aliquots of partially purified enzyme to 2% w/v polygalacturonate solution in 20 mM acetate buffer, pH 4.8, at 25 °C. In the condition used, oligomers with the desired DP were obtained by quenching (100 °C) the enzymatic hydrolysis after 60 min.

After the solution had been boiled for 10 min, the oligomers were precipitated by the addition of 4 volumes of 96% ethanol and subsequently redissolved in water. Separation in low molecular weight (LMW) and high molecular weight (HMW) fractions was achieved by precipitation with 17% aqueous ethanol, following the protocol reported by Spiro et al. (16). After centrifugation, the LMW fraction was obtained in the supernatant, whereas the HMW one was isolated as a precipitate. The LMW fraction was subsequently dried under vacuum, washed twice with 96% aqueous ethanol, dissolved in water, and freeze-dried. The HMW fraction was washed

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twice with 50% aqueous ethanol, dissolved in water, and freeze-dried.

**Electrospray Mass Spectrometry.** The characterization of the two fractions was carried out by means of electrospray mass spectrometry. The LMW fraction was analyzed in the sodium salt form, as obtained after the purification procedure applied, whereas the HMW fraction needed further purification. As suggested by Korner et al. (22), the sodium salt form of the HMW oligomers was converted to their ammonium form by cation exchange chromatography. This step was crucial for the obtainment of a well-resolved mass spectrum. The mass spectra were recorded on an API-1 PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray source and connected to a syringe pump for sample introduction. The instrument was calibrated using a polypropylene glycol (PPG) mixture ( $3.3 \times 10^{-5}$  M PPG 425,  $1 \times 10^{-4}$  M PPG 1000, and  $2 \times 10^{-4}$  M PPG 2000), 0.1% (v/v) acetonitrile, and 2 mM ammonium formate in 50% (v/v) aqueous methanol. The samples were dissolved in 50% (v/v) aqueous acetonitrile/0.63  $\times 10^{-4}$  M ammonium acetate at a final concentration of 2 mg/mL. The spectra were recorded in the negative mode with the electrospray voltage set at  $-5200$  V and the orifice potential at  $-50$  V, using a scan step size of 0.1 amu. The sample flow rate was 5  $\mu\text{L}/\text{min}$ .

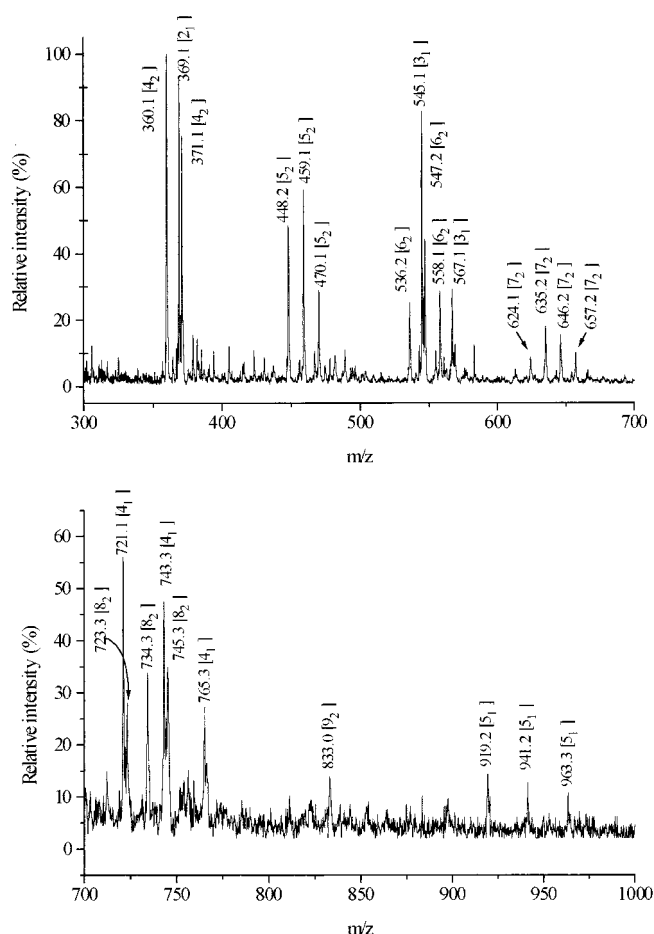
**Circular Dichroism.** Conformational characteristics in the presence of divalent cation interaction were investigated by means of CD using a Jasco J-710 spectropolarimeter. Oligomers ( $[\text{COOH}] = 1 \times 10^{-3}$  M) for CD measurements were always dissolved in 0.1 M  $\text{NaClO}_4$  to avoid an early precipitation upon divalent cation addition; perchlorate salts were used to ensure the suitable optical transparency in the 190–250 nm wavelength range.

## RESULTS AND DISCUSSION

**Oligomer Characterization.** The study described in this paper was focused on two oligogalacturonide fractions containing LMW and HMW species, respectively. Figures 1 and 2 show the mass spectra of the two fractions. The LMW fraction contained oligomers characterized by a DP ranging from 2 to 9. The oligosaccharides formed either singly charged or doubly charged negative ions (Figure 1), and the multiplicity of peaks was due to the presence of sodium as counterion. The evaluation of the concentration of reducing ends gave an average number of galacturonic acid residues per molecule equal to six. A quantitative estimation of the relative amount of each oligomer in the mixture was not possible due to the fact that in this range of molecular weight mass peak intensity decreases with the increasing molecular weight of the oligomer, as also observed for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins (23).

The mass spectrum of the HMW fraction (Figure 2) is well resolved, except for the regions around  $m/z$  354, 530, and 707, where the overlapping of peaks impeded full resolution and consequently their complete assignment. The spectrum showed the presence of oligosaccharides with DP from 7 to 37, but the species more represented the range from DP 8 to 31. The oligosaccharides are present as multiply charged ions, deriving from the loss of at least 3 and up to 10 protons, the amount of charges increasing with the molecular weight. In Figure 2 all of the assigned  $m/z$  values are reported; the corresponding oligomers are indicated only for the series of ions bearing four charges.

It is interesting to point out that the oligomers in the LMW fraction are present in both carboxyl and sodium carboxylate forms, whereas those in the HMW fraction are present exclusively as carboxyl forms. By raising the orifice potential to  $-90$  V, the signals present in Figure 2 diminished in intensity, while peaks corre-

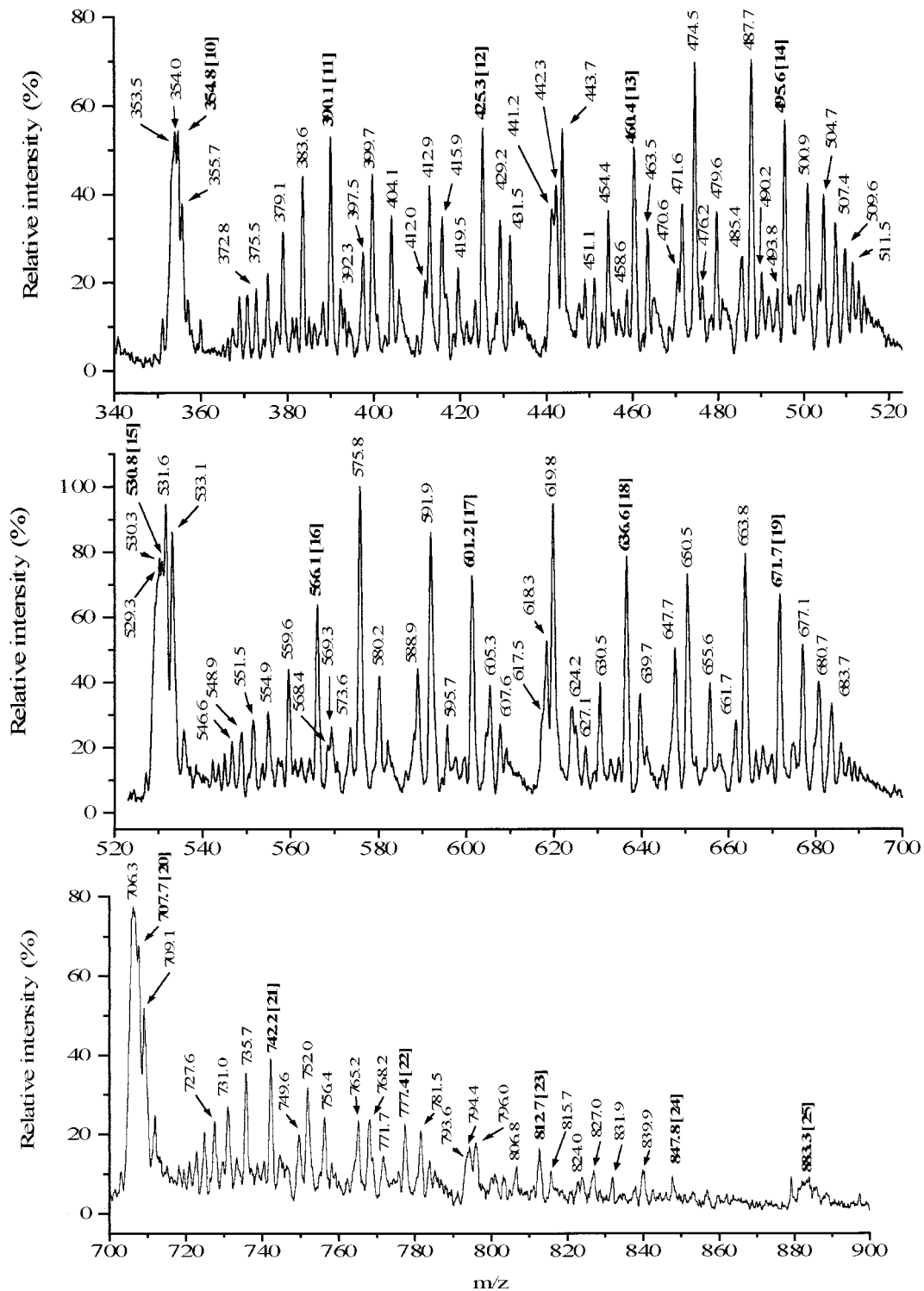


**Figure 1.** Negative mode electrospray mass spectrum of the LMW fraction. The assignment and the charge are indicated in square brackets (e.g., [4<sub>2</sub>] indicates an oligosaccharide with DP 4 and bearing two negative charges).

sponding to DP from 2 to 6 appeared, thus indicating that the HMW fraction contained a small amount of short-chain oligosaccharides (data not shown). It was previously demonstrated that the ESI-MS of cyclic molecules with DP 17–24 could be used for quantitative estimations (24). Therefore, a quantitative estimation of each oligomer in the HMW mixture was attempted. Although some uncertainty was present due to substantial overlapping of peaks, the average DP was calculated to be 19. The determination of reducing-end concentration led to an average number of GalA residues equal to 18 per oligomeric chain. The two values found independently are in very good agreement.

An evaluation of the molecular weight dispersity of the LMW fraction was also attempted by means of gel permeation chromatography (GPC) on a Biogel P2 column. However, the analysis of the compounds eluted in the exclusion volume by means of electrospray mass spectrometry revealed the presence of dimers and trimers, together with oligomers of higher DP, therefore indicating the presence of aggregates. For these reasons, the quantification of the molecular weight distribution was not attained by GPC, but all of the oligomeric samples obtained were always characterized by ESI-MS. As desired, the LMW fraction mainly contained molecules exhibiting a DP < 10, whereas the HMW fraction was composed mainly of oligomers having DP > 10.

**Divalent Cation Interactions with HMW Oligomers.** The conformational behavior of both LMW and

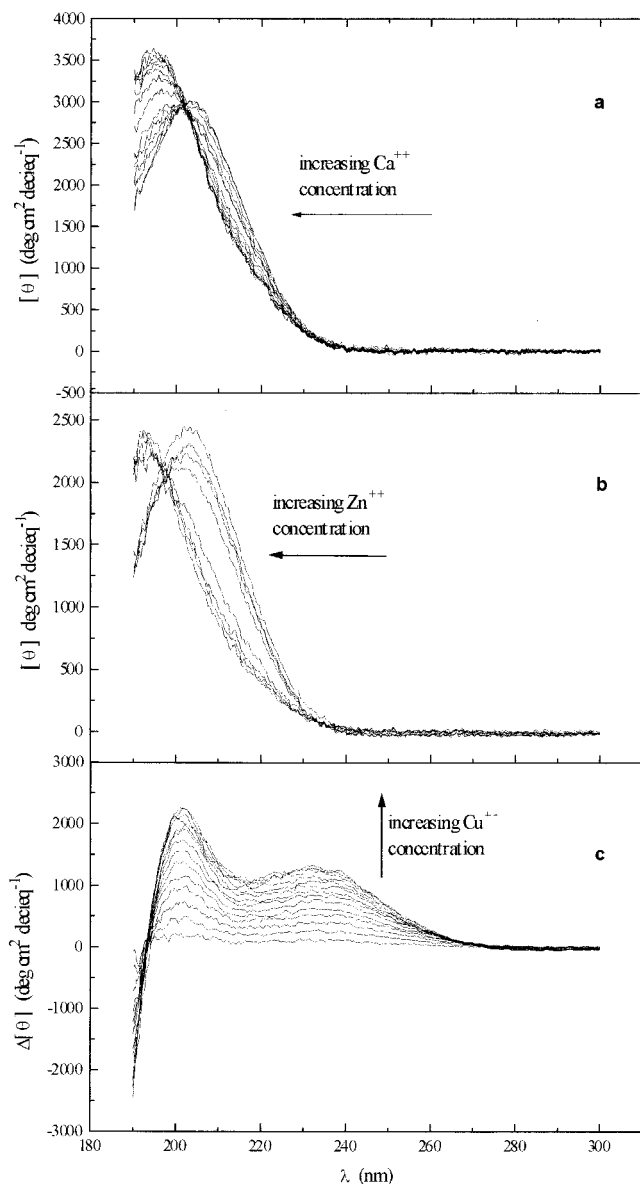


**Figure 2.** Negative mode electrospray mass spectrum of the HMW fraction. The numbers in bold indicate the  $m/z$  values and the corresponding assigned DP (in square brackets) of oligomers bearing four negative charges.

HMW oligomer fractions were investigated in the presence of different divalent cations. Previous investigations by means of EXAFS spectroscopy on  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$  polygalacturonate gels (15) led to the determination of the structural parameters characterizing the geometry of the complex between carboxylate groups on the polymeric backbone and cations. In fact, it was possible to define both distances and coordination numbers relative to the first divalent cation complexation shell. This information was used as a constraint in the molecular modeling calculation to obtain a

detailed model of the conformationally ordered domains in aqueous polygalacturonate gels. Besides calcium ions, copper and zinc ions were investigated because their complexes with molecules containing carboxylate groups have a more defined geometry (square planar, pyramidal, or octahedral) with respect to calcium ions. The same divalent cations were selected in the present investigation in order to compare the conformational characteristics of the polymer, both in dilute solution and in gel, with those exhibited by oligomeric molecules.

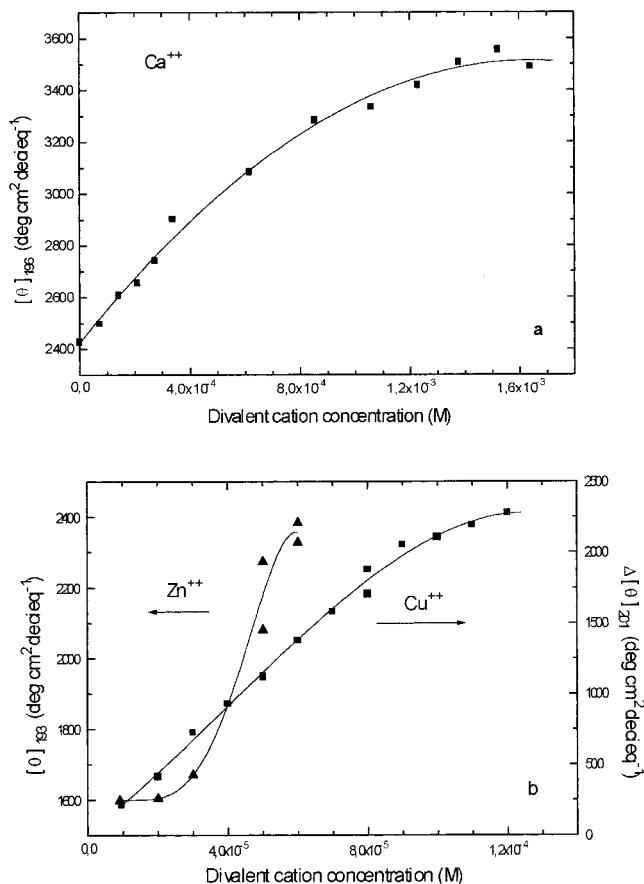
The interaction of divalent cations with both LMW



**Figure 3.** CD spectra of HMW fraction obtained upon increasing divalent cation concentration: (a)  $\text{Ca}^{2+}$  addition; (b)  $\text{Zn}^{2+}$  addition; (c)  $\text{Cu}^{2+}$  addition.

and HMW oligogalacturonide fractions was investigated by means of CD. CD spectra of HMW oligomers in the presence of increasing amounts of  $\text{Ca}^{2+}$  ions are shown in Figure 3a. The presence of an isodichroic point suggested the existence of an equilibrium between two conformationally different forms. The first one pertained to the pure  $\text{Na}^+$  form of the oligomers, whereas the second one was obtained upon addition of  $\text{Ca}^{2+}$  ions (R ratio max = 2.6 mol of  $\text{Ca}/\text{mol}$  of  $\text{COO}$ ). The addition of  $\text{Zn}^{2+}$  ions to the sodium form of oligomers caused a similar CD behavior (Figure 3b). However, the intensity of the low-wavelength CD band was different from that obtained in the presence of calcium ions. This difference can be attributed to the different electronic structure of the complex between carboxylate groups of galacturonic residues and  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ , respectively (15, 25).

Chiro-optical experiments were also carried out to investigate copper complexation. Copper complexes exhibited a more complex CD spectrum due to the presence of a charge-transfer band at  $\sim 240$  nm that partly superimposed to the carboxylate group absorp-

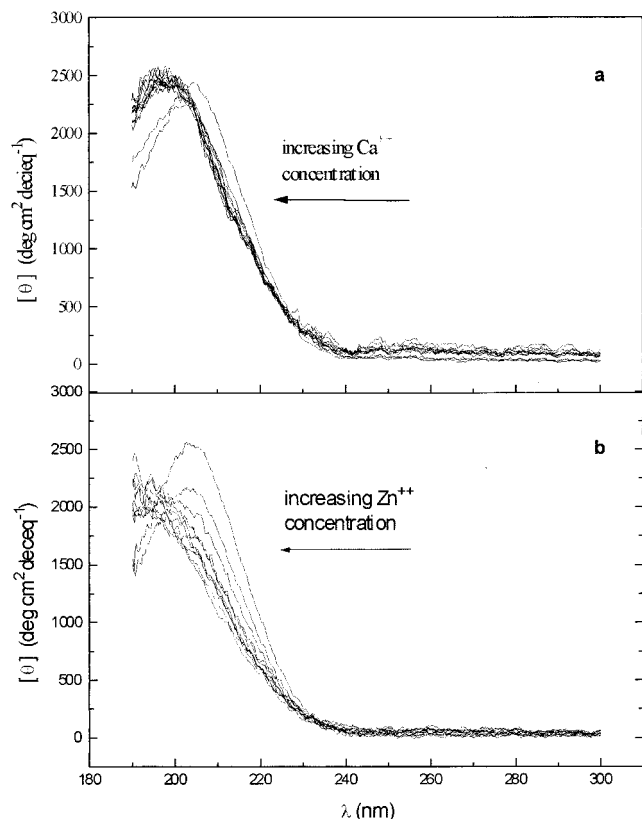


**Figure 4.** Molar ellipticity variation of HMW fraction obtained as a function of increasing divalent cation concentration: (a)  $\text{Ca}^{2+}$  addition; (b)  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  addition.

tion. Therefore, the CD spectra of HMW oligomers are reported as difference spectra after subtraction of the spectrum of pure  $\text{Na}^+$  oligomers (R ratio  $[\text{Cu}^{2+}]/[\text{GalA}] = 0$ ) (Figure 3c). The increase of the signal intensity at  $\sim 240$  nm indicated the binding of copper ions into asymmetric sites on the oligosaccharidic chain, whereas that of the band at  $200$  nm could be related to the ion-driven conformational change of the saccharidic backbone, as reflected by carboxylate group absorption.

The comparison of the effects due to different cations is reported in parts a and b of Figure 4, where the molar ellipticity variation at a given wavelength is reported as a function of the concentration of added cations. It is worth mentioning that the experimental data were obtained in a divalent cation concentration range which did not produce oligomer precipitation. The plots in Figure 4 clearly show that both  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  are by far more efficient than  $\text{Ca}^{2+}$  in producing the conformational transition in the HMW oligomeric fraction. In fact, for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  addition the effect on the oligomer conformation was completed at  $[\text{Zn}^{2+}] \approx 5 \times 10^{-5}$  M and  $[\text{Cu}^{2+}] \approx 1.2 \times 10^{-4}$ , whereas for calcium ions the same effect was obtained at  $[\text{Ca}^{2+}] \approx 1.6 \times 10^{-3}$  M.

**Divalent Cation Interactions with LMW Oligomers.** The effect of divalent cation addition on the conformation of LMW oligomers is hereafter discussed for  $\text{Zn}^{2+}$  and  $\text{Ca}^{2+}$  ions.  $\text{Cu}^{2+}$  ions behaved very alike to  $\text{Zn}^{2+}$  ions. CD spectra are similar to those obtained for the HMW fraction (Figure 5). However, the intensity of the dichroic bands, which appeared after divalent cation addition, is lower than that obtained for HMW oligo-

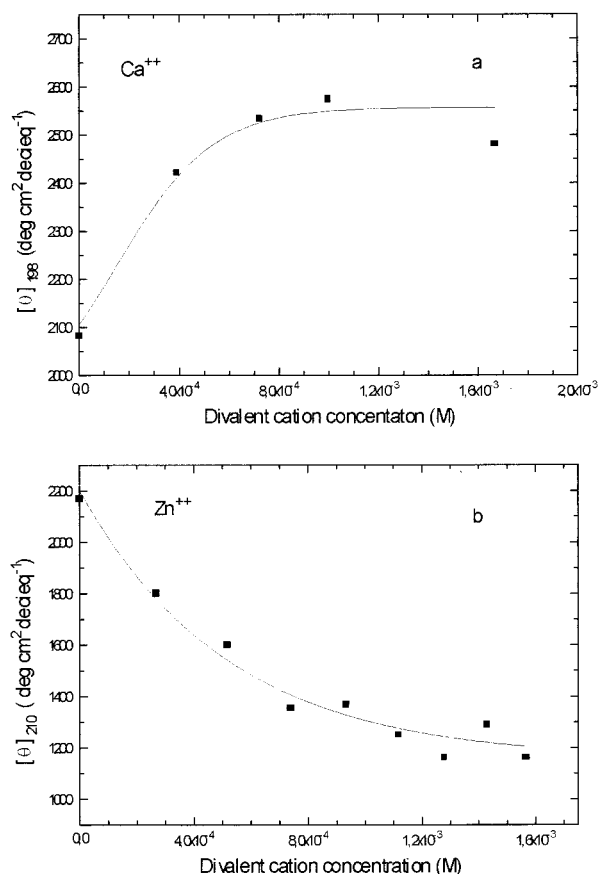


**Figure 5.** CD spectra of LMW fraction obtained upon increasing divalent cation concentration: (a)  $\text{Ca}^{2+}$  addition; (b)  $\text{Zn}^{2+}$  addition.

mers. This effect could be attributed to end effects, which, due to the presence of shorter chains, introduced larger structural disorder for the LMW than for the HMW fraction.

The most interesting result was observed when CD data were plotted as molar ellipticity at a given wavelength as a function of divalent cation concentration, as shown in Figure 6. The conformational transition obtained for  $\text{Ca}^{2+}$  addition was completed at  $[\text{Ca}^{2+}] = 1 \times 10^{-3}$  (Figure 6a); this value was slightly lower than that obtained for the HMW fraction. However, it must be stressed that the percentage of efficient binding sites with respect to the total number of carboxylate groups in LMW oligomers was lower than that of HMW compounds due to the higher contribution of end effects. When interacting with the LMW fraction,  $\text{Zn}^{2+}$  ions induced (Figure 6b) a conformational effect in the same concentration range as  $\text{Ca}^{2+}$  ions. Therefore, the high efficiency of  $\text{Zn}^{2+}$  ions observed when using the HMW fraction was lost in the interaction with oligomers exhibiting an average DP < 10.

**Conclusions.** All of the divalent cations investigated are able to induce a conformational transition in both LMW and HMW oligogalacturonide fractions. The conformational change observed may be attributed to the transition from a rather disordered state, typical of the sodium salt form of the oligomers at an ionic strength of 0.1 M  $\text{NaClO}_4$ , to an ordered secondary structure. As a matter of fact, CD experiments carried out with  $\text{Na}^+$  oligogalacturonides as a function of temperature did not show any feature typical of the occurrence of an order-to-disorder conformational transition, thus confirming the presence of a conformationally disordered state (data not shown). The ordered secondary structure obtained upon divalent cation addition may be identical, or at



**Figure 6.** Molar ellipticity variation of LMW fraction obtained as a function of increasing divalent cation concentration: (a)  $\text{Ca}^{2+}$  addition; (b)  $\text{Zn}^{2+}$  addition.

least very similar, to the one responsible for the formation of ordered domains in polygalacturonate gels, which is characterized by a  $2_1$  symmetry (7).

It is worth mentioning that the specific structural details of complexes formed with different divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$ ) may be significantly different. An investigation of these fine structural features is being carried out in our laboratory by means of X-ray absorption spectroscopy.

In conclusion, the investigation carried out in this study showed that the efficiency of the conformational transition process depended both on ionic species and on the molecular weight of the oligogalacturonide molecules. This observation should be taken into consideration in physiological and biochemical studies aimed at the clarification of the role of oligogalacturonides as elicitors of plant defense mechanisms. In fact, it is worth mentioning that in vivo a number of different ionic species may play a role similar to that exhibited by  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ .

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