COMPLEXATION OF POLY(Lys-Ala) AND POLY(Lys-Ala-Ala-Ala) WITH PECTINS AND POTASSIUM OLIGOGALACTURONATES

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The selected model polypeptides poly(Lys-Ala) and poly(Lys-Ala-Ala-Ala) underwent a helix-forming interaction with potassium pectates and pectinates of various esterification degree (*E*) and with some potassium oligogalacturonates (n = 2—5, 9, 13). Formation of the complex quantitatively monitored by a circular dichroic measurement showed that the specific band distribution of charged side chains of lysine units at the surface of helical structure does not constitute grounds for the local mode of interaction. Potassium pectinate of esterification degree *E* 57%, corresponding to polypeptides by charge density, does not reveal enhanced complexation values. The complex-forming efficacy continuously decreases with the increase of the esterification degree of pectin. Saturation of charges of the polypeptide was achieved predominantly by the spatial action of the superhelical structure of D-galacturonate chains.

Intermolecular interactions of oppositely charged polyelectrolytes forming characteristic complexes are regarded models for biological structures and also materials for industrial and medicinal usage[1,2]. Our previous papers[3-5] dealt with the study of complex formation of potassium pectate and pectinates (~90% polygalacturonate), respectively, with model polypeptides poly(L-Lys) and poly(Lys-Ala-Ala). A considerable change of the secondary structure of the polypeptide in the sense of transition of the charged coil → α-helix at complexation is sensitively reflected in the circular dichroism (CD) spectra, which made it possible to quantify the complexation efficiency. Based upon the estimated stoichiometric relation 1 : 1 between cationic and anionic groups in the complex a spatial structure model of the complex was proposed in which the core of α-helical polypeptide was wound up by the superhelical structure of the polysaccharide. The increase of esterification degree is associated with a decrease of the complexation efficiency due to forced deformation of the pectin chain in the sense of the compression of its superhelix turns. The principal factor determining the effectiveness of interaction between acid polysaccharides and basic polypeptides is the spatial and charge compatibility of the complex components.

The model polypeptides employed were characterized by a different density of the lysine units. The poly(Lys-Ala), prepared for this purpose, represents from this viewpoint a mean polypeptide between poly(L-Lys) and poly(Lys-Ala-Ala), but the spatial arrangement of lysine units is different. Whilst this distribution at the cylindrical surface of the helical structure of the molecule was more or less regular in the preceding experiments, the side chains of lysine form two parallel, slightly mounting turn bands, when employing poly(Lys-Ala). Similar spatial structure as poly(Lys-Ala) has the poly(Lys-Ala-Ala-Ala) molecule containing a half amount of lysine units. The side chains of lysine form only one turn band of the same geometric parameters, i.e. right-handedness, 11 units per turn, pitch 6·5 nm. In continuation of our study it seems interesting to investigate conditions for formation of complexes with these specific structures.

EXPERIMENTAL

Samples of sequentially regular polypeptides poly(Lys-Ala).HBr and poly(Lys-Ala-Ala-Ala)-HBr were synthesized according to[6]. The relative molecular masses estimated from the sedimentation equilibrium by the method described in ref.[7] were as follows: poly(Lys-Ala). HBr 8 800, poly(Lys-Ala-Ala-Ala). HBr 6 500. The lysine content in freeze-dried preparations for the complexation experiments was determined from the concentration of Br^- ions by the potentiometric titration with $AgNO_3$ (2 mmol l^{-1}).

Pectin preparation of various esterification degree of carboxyl groups with methanol: commercial citrus pectin (Genu Pectin, Medium Rapid Set, Type A, Københavns Pektinfabrik, Denmark) was purified by the method described in our previous paper[3]. The highly esterified pectin (E 94%) was prepared from the purified pectin by esterification with methanolic H_2SO_4 (1 mol l^{-1}) at 3°C during 3 weeks (ref.[8]). This pectin served as a starting material for the preparation of samples of various esterification degree (Table I). These were obtained by a controlled alkaline deesterification of aqueous solution of highly esterified pectin (1%) with KOH (1 mol . l^{-1}) followed by neutralization and freeze-drying of the solution. The freeze dried samples were thoroughly washed with 60% ethanol and 96% ethanol and air-dried. The potassium oligogalacturonates used in this work were samples, the preparation, purification, characterization and determination of the polymerization degree of which were already published[9,10].

The content of free and total carboxyl groups in pectin, content of partially esterified D-galacturonan in dry matter and the esterification degree (Table I) were determined by precipitation of insoluble copper pectates and pectinates[11,12], and by potentiometric titration with KOH (0·05 mol l^{-1}). The limit viscosity number $[\eta]$ was estimated by means of Ubbelohde viscometer in solution of NaCl (0·15 mol l^{-1}) and sodium oxalate (5 mmol l^{-1}) at 25 ± 0·1°C. The equation by Owens and coworkers was employed to reduce the $[\eta]$ value to relative molecular mass $\overline{M}_r$.

Mixtures for investigating the complexation were prepared from the starting solutions of pectin (potassium pectate and pectinate), or potassium oligogalacturonates of free carboxyl groups concentration 0·6 mmol ($-COO^-$) l^{-1} and from solutions of polypeptides of the same concentration (0·6 mmol ($-NH_3^+$) l^{-1}). Interactions of polypeptides with pectin of various esterification degree, or with potassium oligogalacturonates were examined in two series: *I*) solution of the polypeptide, corresponding to 20, 40, 60, 80, and 100% addition per free carboxyl groups was poured into the solution of either pectin, or potassium oligogalacturonate. Final concentrations of both the pectin and the oligomer were adjusted by dilution to a uniform concentration

0·3 mmol ($-COO^-$) l^{-1} for the whole series. *II*) The solution of either pectin or potassium oligogalacturonate, corresponding to 20, 40, 60, 80, and 100% addition per ($-NH_3^+$) l^{-1} was poured into the solution of the polypeptide. The final concentration of the polypeptide was adjusted to 0·3 mmol ($-NH_3^+$) l^{-1} for the whole series. The CD spectra were recorded with a Dichrograph Mark III (Jobin Yvon, France) in 1 and 5 mm cells at 25°C.

Employed were: a digital potentiometer Radiometer PHM 64 (Denmark), a silver electrode, electrolytic bridge filled with 10% KNO_3 solution, a combined electrode GK 2 401 C (Radiometer), carbonate-free KOH (0·05 mol l^{-1}), and redistilled CO_2-free water.

RESULTS AND DISCUSSION

Conformational and chiroptical features of pectins of various esterifications degree were described in ref.[14]. Table I lists the parameters of maxima of the chiroptic band. The ellipticity value increases with the increasing esterification degree due to a higher value of the esterified form than of that of the ionized carboxyl group. The CD spectra of poly(Lys-Ala) in a neutral aqueous solution, i.e. in a charged coil arrangement, and in an alkaline medium, where after neutralization of the polycation charge a regular helical structure is formed, are presented in Fig. 1.

The CD spectra of mixtures of pectin with poly(Lys-Ala) were recorded in two series – poly(Lys-Ala) was stepwise added, to a constant amount of $-COO^-$ groups of pectin and vice versa, pectin was added to a constant amount of poly(Lys-Ala). The spectra obtained were corrected by subtracting the CD of all pectin present, and in the second series also the CD of the excess of poly(Lys-Ala) in the charged coil arrangement. The CD spectra corrected in this way represent the CD of poly(Lys-Ala) having an equivalent amount of carboxyl counterions required for inter-

TABLE I
Characteristic data of pectin preparations

E^a %	G^b %	$[\eta]$ ml g^{-1}	$\overline{M}_r$	$\Delta\varepsilon$ (nm)
0	89	122	27 100	0·9 (207)
14·6	89	90	21 500	1·2 (204)
25·2	88	91	22 000	1·4 (205)
35·4	90	98	23 000	2·0 (205)
43·1	89	89	21 400	2·4 (206)
57·0	91	101	23 600	2·9 (207)
66·8	92	119	26 500	3·7 (207)
73·7	89	109	25 000	4·5 (209)
82·5	90	134	29 000	7·0 (208)

[a] Esterification degree; [b] content of a partially esterified D-galacturonate in dry matter.

action. Fig. 2 shows such spectra involving pectin of esterification degree 0 and 25%. Samples with 60% addition of poly(Lys-Ala) (curve *3*) evidently differ from the rising trend of $\Delta\varepsilon$ values observed. These samples revealed a most remarkable opalescence observed in solution containing an excess of pectin. The negative $\Delta\varepsilon$ values of the first long-wave dichroic band are higher with samples having pectin in excess when compared with those having poly(Lys-Ala) in excess. On the other hand, the $\Delta\varepsilon$ values in the short-wave region are lower in solutions containing an excess of pectin. Generally speaking, the CD spectra of the series with an excess of pectin and those with an excess of poly(Lys-Ala) differ from each other both in the shape of the spectrum and intensity of the first dichroic band. This finding is distinct from the previous ones[3,4], where a mutual accordance of CD spectra in both series of unesterified pectin (*E* 0%) was observed irrespective of the ion in excess. This agreement indicates a quantitative transition of the polypeptide to a helical conformation in complexation in the ratio of 1 : 1 of the interacting components. Nonetheless, with unesterified pectin (the highest charge density), the formation of complex with poly(Lys-Ala) under investigation is not fully quantitative. According to intensity values of the first dichroic band (corresponding to the $n \rightarrow \pi^*$ electron transition of the amide group) it becomes evident that complexation is more effective with samples containing an excess of pectin. The lowered intensity of dichroic absorption in a short-wave region (corresponding to a part of the $\pi \rightarrow \pi^*$ couplet of the amide group transition) can be partly attributed to contribution of the positive CD of poly(Lys-Ala) remaining in the charged coil arrangement, i.e. not entering the complex. However, the main reason was obviously the remarkable opalescence of systems under study which, as seen mainly with 60% addition of polypeptide, lowers

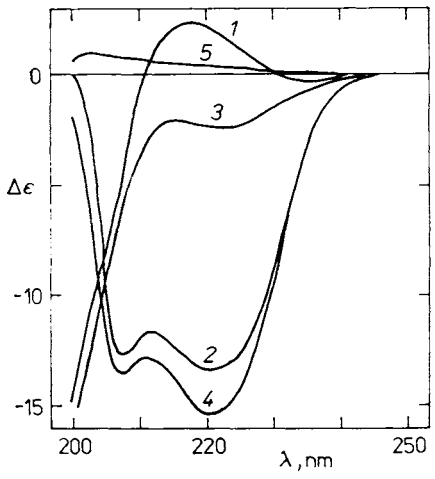

FIG. 1

The CD spectrum of poly(Lys-Ala), ($-NH_3^+$) 0·3 mmol l^{-1}: *1* at pH 7·0, *2* at pH 11·3 and poly(Lys-Ala-Ala-Ala) ($-NH_3^+$) 0·3 mmol l^{-1}, *3* at pH 7·0, *4* at pH 11·3, *5* potassium pectate *E* 0%, ($-COO^-$) 0·3 mmol l^{-1}

the values of dichroic absorption prevalently in the 210 nm region. As found[15], polypeptides with alternating sequence of the charged hydrophobic side chain (Lys-Leu) revealed the presence of a β-sheet structure in aqueous solutions of salts, characterized in the CD spectrum by the negative maximum at 215 nm. The spectral band of the complex observed with 60% addition of polypeptide has a considerably reduced intensity in this region and, therefore, it is difficult to characterize unambiguously the structure of the complex from the shape of the spectrum only.

Intensity of the negative dichroic absorption decreases with the increasing esterification degree (E) of pectin. The experimental CD values at $\lambda = 225$ nm modified in the above mentioned manner and normalized to a uniform concentration of the ion added (i.e. to 0·3 mmol $(-NH_3^+)\,l^{-1}$ for series *I* and 0·3 mmol $(-COO^-)\,l^{-1}$ for series *II*) were employed to calculate the complex-forming efficacy. The CD value of poly(Lys-Ala) obtained at pH 11·3 was taken for a basic measure to evaluate the percentage of efficiency (A). It is presumed that at this pH polypeptide molecule

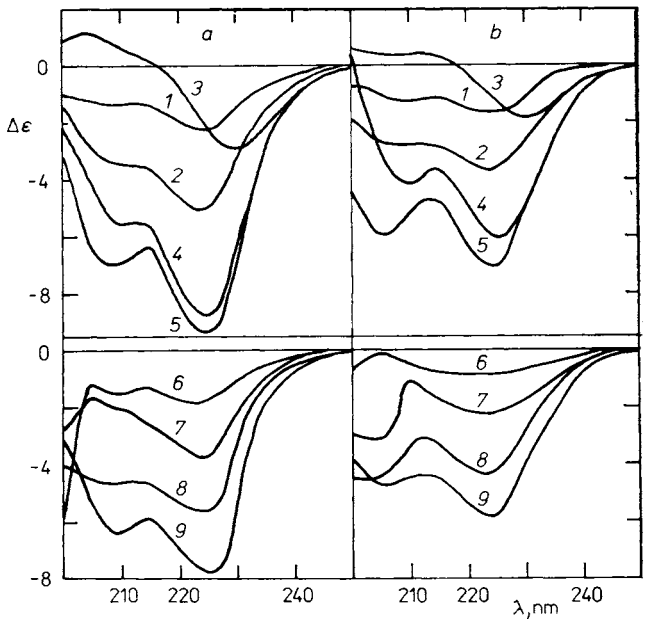

FIG. 2

The corrected CD spectrum of poly(Lys-Ala) in the presence of potassium pectate E 0% (*a*) and potassium pectinate E 25·2% (*b*), $-COO^-$) 0·3 mmol l^{-1}; *1*, *2*, *3*, *4*, *5* solution of pectin containing 20, 40, 60, 80 and 100% of ion-equivalent amount of poly(Lys-Ala); *6*, *7*, *8*, *9* solution of poly(Lys-Ala) ($(-NH_3^+)$ 0·3 mmol l^{-1}) containing 20, 40, 60 and 80% of ion-equivalent amount of pectin

adopts the total helical arrangement ($A = 100\%$). The values obtained are shown in Fig. 3. It can be stated, even at a considerable scattering of the values that the continuous decrease of the complex-forming efficacy is associated with the increase of the esterification degree, i.e. with a decrease of the polysaccharide charge density. With pectin samples of low esterification degree the highest efficiency was achieved at 40% addition of poly(Lys-Ala), with those of high esterification degree at 20% addition of polypeptide. In the case of 60% addition of poly(Lys-Ala), when a very intense opalescence takes place, the values obtained evidently deviate from the given relationship with samples of $E < 50\%$ and are, therefore, not considered.

From the interaction studied so far[3-5] it has been deduced that the lysine and pectin polymers interacted in stoichiometric ratio of their charges to give the resulting complex. It could be presumed that this ratio remains roughly unchanged also in the system with poly(Lys-Ala) under study. Differences in the interaction efficiency in both series can be explained by steric requirements influencing the charge saturation during formation of the complex. With poly(Lys-Ala) in α-helical conformation, where one turn is formed by 3·66 peptide units, the side chains of lysine units are oriented so that each second side chain occurs approximately on the opposite side of the cylindrical surface of the molecular structure. Examination of the spatial model reveals that the shortest vertical distance between $-NH_3^+$ groups of the neighbouring turns is 1·05 nm, thus forming two separated coils of $-NH_3^+$ surface charges. To form a complex, i.e. to transform poly(Lys-Ala) into a helical structure, both the above mentioned coils of surface charges should be saturated. This process is spatially pretentious and may be facilitated by an excess of pectin in solution. Our preceding results[3,4] let us conclude that the saturation proceeds in the sense of

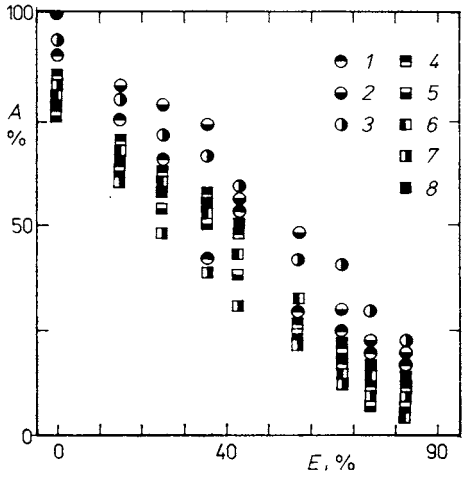

FIG. 3

The complex-forming efficacy A (%) of pectin of various esterification degree (E). Solution of pectin with 20% (*3*), 40% (*2*) and 80% (*1*) of poly(Lys-Ala) and solution of poly(Lys-Ala) with 20% (*7*), 40% (*5*), 60% (*6*), 80% (*4*) and 100% (*8*) of pectin added

surface equilibration of the charge density of the polypeptide and polysaccharide. The situation with poly(Lys-Ala) is somewhat more complicated. The distance between the vicinal carboxyl groups in pectate (E 0%) was found to be 0·437 nm (ref.[16]). Provided a point saturation of charges, the greatest complexation effect would be expected with pectin of esterification degree E 57%, where the mean distance of $-COO^-$ groups is closest to the above-mentioned value 1·05 nm. In this case at the total saturation of charges (100% addition of poly(Lys-Ala)), two pectin chains would form a superhelix around the α-helix of the polypeptide molecule tracing its $-NH_3^+$ surface charges. However, the low values of complexation efficacy of pectin of the esterification degree E 57% indicate different mechanism. The complex-forming electrostatic interaction does not proceed by the point interaction mechanism, but by a spatial saturation of charge densities on molecular surfaces.

The spatial compensation of charges can be achieved by interaction of either one or more pectin chains surrouding the polypeptide molecule. Employing geometric parameters it is possible to deduce for one chain of unesterified pectin that one turn of its superhelix should be composed of 28 galacturonic acid units with a 8·58 nm pitch. The increasing esterification degree of pectin, i.e. the decreasing charge density is associated with compression of the superhelix turns. The limit value is the van der Waals' contact distance ($\sim$1·1 nm), theoretically corresponding to pectin of E 85%. In this case, as seen from Fig. 3, the complexation efficacy decreases to zero value. Considering an interaction with more polysaccharide chains one may calculate that two polysaccharide chains linearly oriented with respect to the axis of the helix, would fully compensate the charge density of the polypeptide at the esterification degree E 26%. Higher esterified pectins would obviously have both chains in a superhelical orientation. This is also the theoretical case with pectin of E 57%, where both turns of the surface $-NH_3^+$ charges would directly be saturated by two polysaccharide chains of the same pitch. Complexation involving more polysaccharide chains (2–8) is less probable because the concentrations of both comples-forming components are too low, and the polysaccharide molecules are by 8 times longer in the average than the mean linear length of the poly(Lys-Ala) α-helix. This statement is also supported by the fact that at esterification degree E 57% an enhanced complexation efficacy was not observed, as would be expected for a simultaneous interaction of two polysaccharide chains.

Complexation leads, similarly as with poly(Lys) and poly(Lys-Ala-Ala), refs[1,2], predominantly to a superhelical structure with one polysaccharide chain belonging to one helical polypeptide chain. The most intense formation of complexes takes place at 60% excess of pectin (Fig. 2). Therefore, it could be assumed that in the initial phase of complexation two polysaccharide chains enter the interaction. The charge unbalance of transition state brings about its rearrangement in the next step. Low esterified pectins of high charge density attract further oppositely charged polyions. The observed opalescence is due to formation of aggregated systems.

On the other hand, the samples containing smaler proportions of pectin, i.e. those with excess poly(Lys-Ala), as well as those containing pectins of higher esterification degree give rise to unaggregated complexes with one superhelical polysaccharide chain.

The polysaccharide superhelix can be either right-handed, i.e. following the spiral rise of surface charges of the poly(Lys-Ala) helix, or left-handed, bridging transversally the spiral rise of the surface charges of the polypeptide helix. To ascertain how effective are the individual interactions, complexations with oligogalacturonates of various chain length were studied. Our previous investigation[3] with poly(Lys-Ala-Ala) showed that the complexation efficacy increased with the increase of the oligosaccharide chain. The highest values were observed when the chain length was approximately equal to the distance between two surface $-NH_3^+$ groups. The distance between the closest $-NH_3^+$ groups of neighbouring turns in poly(Lys-Ala) (1·05 nm) was substantially shorter than that of poly(Lys-Ala-Ala) and was close to the distance of $-COO^-$ groups in the molecules of tri- and tetragalacturonates (0·88 and 1·31 nm, respectively). The distance between two bands of surface $-NH_3^+$ groups in the helical poly(Lys-Ala) is 2·7 nm. Fig. 4 shows the values of complex-forming efficacy obtained from $\Delta\varepsilon$ values at 225 nm for oligomers with n = 2, 3, 4, 5, 9, and 13 by the procedure already described. As seen from Fig. 4 complexation was evident with tri- and mainly with tetragalacturonate. Bridging of the closest $-NH_3^+$ groups belonging to two adjacent turns of the polypeptide becomes effective. The complexation efficacy depends considerably on the mutual ratio of both components. Higher values were found with an excess of the oligosaccharide. The complexation efficacy increases with increasing chain length with oligomers added in more than 50% excess;

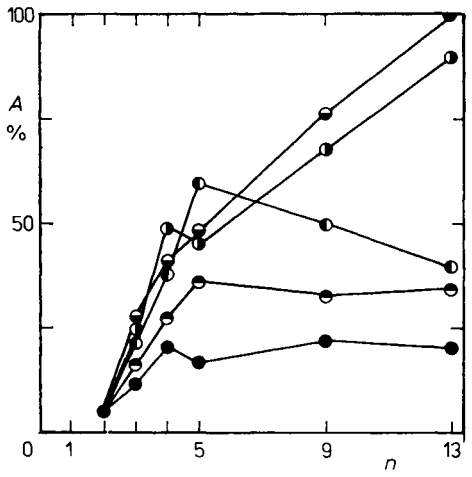

FIG. 4

The complex-forming efficacy A (%) of oligogalacturonates of various polymerization degree (n) at different ratios of components. The values are normalized to the same concentration of poly(Lys-Ala). The solution of oligogalacturonates with 20% ◑, 40% ◒, 60% ◐, 80% ◓ and 100% ● of poly(Lys-Ala) added

with lower amounts of oligomers of DP 9 and 13 the complex forming-efficacy significantly decreased.

The structurally more simple analogue of poly(Lys-Ala), the sequentially homogeneous poly(Lys-Ala-Ala-Ala) in a helical conformation has only one spiral turn of lysine surface charges. The CD spectrum of the polypeptide itself (Fig. 1) indicates a higher content of the helical form even in a neutral solution. Pectins of *E* 0, 35·4, and 57% were employed for investigation of the complex-forming interaction. The results of measurements were processed by the afore-mentioned method. The individual complex-forming efficacy (*A* 100% for the peptide at pH 11·3) was calculated from the $\Delta\varepsilon$ values at 225 nm. Fig. 5 shows a considerable difference for the individual stoichiometric ratios. The highest complexation values were observed with the highest excess of the polypeptide (20% addition of the pectin). These values decrease gradually with further addition of pectin. The complex-forming efficacy values with pectin of *E* 57% were lower than those with other pectins and consequently, a direct unambiguous point saturation of charges did not take place. The relatively highest efficacy was observed with pectin of *E* 35·4%. A wide distribution of efficacy values has not been observed thus far, probably due to the fact that a stoichiometric ratio 1 : 1 of $-COO^-$ and $-NH_3^+$ charges in the complex could not be reached. The interaction proceeds with a local excess of $-COO^-$ against $-NH_3^+$. This unbalance of charges brings about an aggregation of further molecules. Addition of 100% amount of pectin leads to formation of unmeasurable aggregates.

It is obvious that poly(Lys-Ala-Ala-Ala) adopts helical conformation more easily than poly(Lys-Ala), and therefore, it probably forms complexes with pectins in another way and of ambiguous spatial structure.

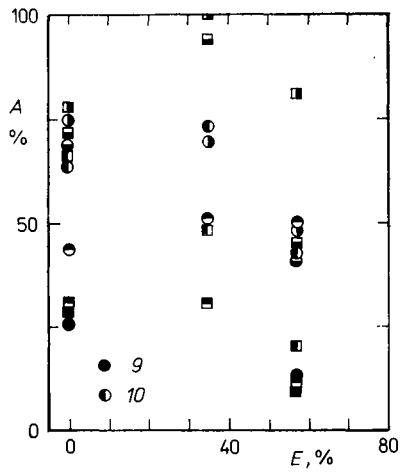

FIG. 5

The complex-forming efficacy *A* (%) of pectin of esterification degree *E* 0, 35·7 and 57·0%. Solution of pectin with varying amounts of poly(Lys-Ala-Ala-Ala). 60% (*10*), 100% (*9*). The other points correspond to the percentage values given in Fig. 3

The results presented let us presume that the interaction in which the polyanionic molecule follows a right-handed orientation of the surface polypeptide ions is more favourable and prevails when compared with the transversal saturation of side charges of lysine. In the complex with poly(Lys-Ala) the unesterified pectin molecule probably occupies a position binding simultaneously both surface bands of positive charges. This is allowed by geometric parameters described and by the linear charge density of the pectate, which is adequate to saturation of both turns. The esterified pectins show a deviation from the parallelism, charge concentration was attained by lowering the superhelix pitches.

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