FORMATION OF THE POLY(Lys-Ala-Ala) COMPLEX WITH PECTIN OF VARIOUS ESTERIFICATION DEGREE

Slavomír BYSTRICKÝ[a], Rudolf KOHN[a], Tibor STICZAY[a]
and Karel BLÁHA[b]

[a] *Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava and*
[b] *Institute of Organic Chemistry and Biochemistry,*
Czechoslovak Academy of Sciences, 166 10 Prague

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The helix-forming interaction of poly(Lys-Ala-Ala) with potassium pectinates of various esterification degree (E 0—87%) was quantitatively investigated by circular dichroism spectra. The helix-forming effect of pectinates is significant even at a low density of carboxyl anions in the molecule at a high esterification degree. The mutual complementarity of linear charge density of both interacting polyions plays a decisive role. The charge densities of unesterified pectate and helical poly(Lys-Ala-Ala) are very close. Orientation of the potassium pectate macromolecule (E 0%) in the proposed spatial model is parallel to the axis of poly(Lys-Ala-Ala) α-helix. Saturation of the polypeptide charge with pectinates of an esterification degree $E > 0\%$ is achieved through formation of a superhelical structure of the pectinate with a turn density corresponding to the equivalence of charge densities of both interacting components in the complex.

An attractive interaction of cationic polypeptides with anionic polysaccharides in solution results in formation of complexes under a considerable change of the polypeptide conformation observable in circular dichroism spectra. Much attention has been paid to investigation of interactions with mucopolysaccharides[1-3] and heparin[4,5]. As found, glycosaminoglycans containing mainly sulfate anions induce, as a rule, an α-helical structure of poly(L-lysine)*. In contrast, hyaluronic acid and desulfated chondroitin sulfate, containing only carboxyl groups, do not induce the helical structure of poly(lysine); refs[1,2]. The strength and stoichiometry of interaction generally depend on the length of side-chains of the polypeptide, on the degree and position of sulfatation, and eventually, on the effect of carboxyl groups present. Reason for the absence of the α-helix directing interaction of hyaluronic acid and chondroitin was originally considered the absence of sulfate ions[2]. The helix-forming interaction was later also observed with polycarboxylates and polyphosphate[6-8].

Our preceding paper[9] dealt with the effect of pectin polyanions on conformation of poly(lysine). A fully deesterified pectin quantitatively forms an α-helical complex in a stoichiometric 1 : 1 ratio of cationic to anionic groups. The α-helix directing

* All amino acids in this paper refer to L-configuration.

effect decreases with the decrease of linear charge density, *i.e.* with an increase of esterification degree of pectin. The presumed structure of the complex consists of the polypeptide core in an α-helical conformation, which is probably wound up by a superhelical structure of the acidic polysaccharide. One superhelix turn characterized by 3·4 nm pitch contains roughly 23 D-galacturonic acid units. The successive decrease of charge density of pectin resulted in an stepwise compression of turns of the superhelix structure. The model is based upon the observed mutual saturation of charges. Proof of the validity of this model, as well as the detailed evaluation of factors determining formation of the complex require to study this interaction with other model polypeptides. This paper concerns the interaction of pectin of various esterification degree with a sequentially regular poly(Lys-Ala-Ala) characterized by a three times lower charge density in comparison with the hitherto investigated poly(lysine).

EXPERIMENTAL

Samples of sequentially regular poly(Lys-Ala-Ala)hydrogen bromide (*I* and *II*) were synthesized according to[10]. Their relative molecular masses estimated from the sedimentation equilibrium of ultracentrifugation[11] were found to be 7 580 ± 300 and 6 800 ± 500, respectively. The lysine content in freeze-dried preparations was determined from the concentration of $Br^{(-)}$ ions *via* potentiometric titration with $AgNO_3$ 2 mmol l^{-1}.

The pectin preparations of various esterification degree (E) of carboxyl groups with methanol were obtained from a purified commercially available citrus pectin (Genu Pectin, Medium Rapid Set, Type A, Københavns Pektinfabrik, Denmark) by a procedure described in the preceding paper[9]. Samples 1–4 (Table I) were prepared by a partial alkaline deesterification of the starting pectin ($E \sim 67\%$) in suspension, in 60% ethanol, with a dilute potassium hydroxide solution. The remaining samples (5–12) were obtained by the same procedure from a highly esterified pectin preparation ($E \sim 95\%$) (for details see[9]). The highly esterified pectin was prepared by esterification of the commercial sample of pectin with methanolic H_2SO_4 1 mol l^{-1} at 3°C for 3 weeks[12].

The content of free and total carboxyl groups in pectin, that of polyuronide (partially esterified D-galacturonan) in the dry substance and the esterification degree E were determined by the precipitation method of copper pectates and pectinates[13,14]. The content of free carboxyl groups in pectin preparations of high esterification degree ($E \geqq 71\%$) was alkalimetrically determined by a potentiometric titration with KOH 0·05 mol l^{-1}; the total carboxyl groups were even here determined with the Cu-pectate method. The content of copper in the coagulate was chelometrically estimated by titration with a Complexon IV 0·01 mol l^{-1} in a weakly ammoniacal medium, murexide being the indicator. The point of equivalence was spectrophotometrically determined (interference filter Zeiss, Jena, IF 600 nm). The limit viscosity number $[\eta]$ was determined by means of Ubbelohde viscometer at 25·0 ± 0·1°C in aqueous NaCl 0·15 mol l^{-1} and sodium oxalate 0·005 mol l^{-1}. The $[\eta]$ values were reduced to relative molecular mass ($\bar{M}_r$) according to Owens equation[15] conventionally most employed.

Mixtures of pectin with poly(Lys-Ala-Ala) were prepared from the starting potassium pectate and pectinate solutions, respectively, at a total carboxyl groups concentration ($-COO^{(-)} + -COOCH_3$) 0·600 mmol l^{-1} and poly(Lys-Ala-Ala)hydrogen bromide in a ($-NH_3^{(+)}$) 0·600 mmol l^{-1} concentration. The solution of potassium pectate or pectinate was added to

poly(Lys-Ala-Ala) solution in such a way that the concentration of ($—COO^{(-)} + —COOCH_3$) was 20, 40, 60, 80, and 100% per ($—NH_3^{(+)}$). The final concentration of systems under study was always uniformly adjusted to a ($—NH_3^{(+)}$) 0·300 mmol l^{-1} concentration. The pH values of these mixtures varied within 5·5 to 6·4 depending on the ratio of both components and esterification degree of pectin *E*. The circular dichroism spectra were measured with a Jobin Yvon (France), Mark III dichrograph in 1 and 0·5 mm-cells at 25°C.

Digital potentiometer Radiometer PHM 64 (Denmark), silver electrode, electrolytic bridge filled with a 10% KNO_3 solution, glass electrode G 222B, saturated calomel electrode K 401 (Radiometer), carbonate-free KOH 0·05 mol l^{-1} and redistilled carbon dioxide-free water were employed.

RESULTS AND DISCUSSION

Chemical structure of pectin, conformation of its macromolecule and circular dichroism (CD) of potassium pectinates of various esterification degree of carboxyl groups *E* were described in our preceding paper[9]. The low-intensity positive Cotton effect in the 205 nm region was only little shifted towards shorter wavelengths upon the increasing esterification degree of pectin. The CD spectrum of poly(Lys-Ala-Ala) in neutral aqueous solution in a "charge-coil" arrangement is shown in Fig. 1.

The CD spectra of mixtures of potassium pectate or pectinate with poly(Lys-Ala-Ala) were recorded at a various ratio of both components; the investigated solution contained an equivalent amount, in most cases an excess of ($—NH_3^{(+)}$) groups per

TABLE I
Characteristic data of pectin samples

Sample	Esterification degree, *E*, %	*G*[a] %	$[\eta]$ ml g^{-1}	$\overline{M}_r$
1	0	89	117	26 000
2	21·6	86	182	37 000
3	44·1	83	283	51 000
4	56·3	86	324	56 000
5	65·3	91	119	27 000
6	71·4	91	114	26 000
7	74·7	91	128	28 000
8	77·9	91	119	27 000
9	79·8	91	159	33 000
10	82·6	91	131	29 000
11	86·7	91	142	30 000
12	87·1	91	158	33 000

[a] Content of partially esterified D-galacturonan in dry substance (H^+ form).

($-COO^{(-)}$) groups (see the experimental section). To normalize the CD spectra to a unified concentration of ($-COO^{(-)}$) groups, the circular dichroism of the excess poly(Lys-Ala-Ala) (conformation "charge-coil") was first of all subtracted in regard to the excess of ($-NH_3^{(+)}$) groups against the amount of ($-COO^{(-)}$) groups in solution, as well as the intrinsic circular dichroism of the pectin added. (Considering the preceding results we believe the spectrum of pectin does almost not change on interaction with the polypeptide).

The corrected spectrum presents, therefore, the circular dichroism of that quantity of poly(Lys-Ala-Ala), in which its cationic ($-NH_3^{(+)}$) groups and anionic ($-COO^{(-)}$) groups of pectin in solution are equivalent. The corrected spectra were then normalized to the unified concentration of carboxyl groups ($-COO^{(-)}$) 0·300 mmol l^{-1}. Samples of low-esterified pectin revealed a noticeable opalescence, which successively disappeared with increasing esterification degree of pectin. The mixed solutions with pectin of $E = 56\%$ were already clear.

Fig. 1 shows the corrected CD spectra of poly(Lys-Ala-Ala) in the presence of potassium pectinate for some selected samples of pectin of various esterification degree E. Concentration of both components of mixtures, *i.e.* ($-NH_3^{(+)}$) groups of the polypeptide and the total carboxyl groups of pectin ($-COO^{(-)} + -COOCH_3$) was identical, 0·300 mmol l^{-1}. Spectra characteristic of two peaks in the negative region indicate the presence of the polypeptide in an α-helical conformation. The dichroic band in the 225 nm region associated with the $n \rightarrow \pi^*$ electronic transition characterizes the extent of the helix-forming interaction. Intensity of this band decreases with the increasing esterification degree of pectin; this evidences the effectiveness of only ionized carboxyl groups ($-COO^{(-)}$) in the complex-forming interaction. The second dichroic band at about 208 nm belongs to the long-wavelength (negative) part of the exciton-couple of the optically active $\pi \rightarrow \pi^*$ electronic transition. The sample with the unesterified pectate (E 0%) is characteristic of only a short-

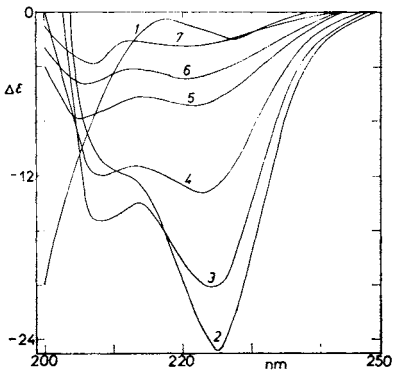

FIG. 1

The corrected CD spectrum of poly(Lys-Ala-Ala) and its complexes with pectin of various esterification degree E. ($-NH_3^{(+)}$) 0·3 mmol l^{-1}; ($-COO^{(-)} + -COOCH_3$) 0·3 mmol l^{-1}; *1* poly(Lys-Ala-Ala), *2*, *3*, *4*, *5*, *6*, *7* poly(Lys-Ala-Ala) with addition of potassium pectinate of $E = 0$, 21·6, 44·1, 65·3, 74·7, and 82·6%, respectively

-wavelength shoulder at an intense dichroic band of the $n \rightarrow \pi^*$ transition. The corrected CD spectrum of polypeptides interacting with pectinates of esterification degree $E \geqq 44\%$ has already a typical shape for α-helical structure.

The special shape of CD curves with an insignificant short-wavelength negative dichroic band, observed with low-esterified pectinate complex can not be classified into known manifestations of regular protein structures[16]. Certain similarity could be encountered with the CD curve for β-structure. The sequence copolymer poly-(Lys-Ala-Ala) does not, of course, form a β-structure[17]. The β-structure is indicative of only one negative dichroic band with the maximum shifted towards lower wavelength (~215 nm), ref.[18]. In contrast, maximum of the negative dichroic absorption in our samples was shifted towards higher wavelengths with the decreasing esterification degree. A possible rationalization for the unusual shape of the CD spectrum could be the fact that the reduced intensity in the short-wavelength region of the helical conformation spectrum could be due to a considerable opalescence of samples. This phenomenon was alredy observed[19] with interaction of glycosaminoglycanes with polyarginine. Extent of the complex-forming interaction was investigated at 225 nm, *i.e.* at reference values of ellipticity ($\Delta\varepsilon_{225}$), where the effect of opalescence is no more so significant. The correction on the CD spectrum of pectin is at this wavelength very little. The ellipticity values, normalized to the unified concentration of (—$COO^{(-)}$) groups of the pectinate (0·3 mmol l^{-1}) were employed for correlation of the efficiency of the helix-forming interaction (B) and the linear charge density of the pectinate (Fig. 2). The value B expresses the per cent of poly(Lys-Ala-Ala) passing into the helical form in the complex at a normalized addition of ionized carboxyl groups. The linear charge density is directly proportional to the expression $1 - E/100$, where E stands for the esterification degree of pectin. The effectiveness

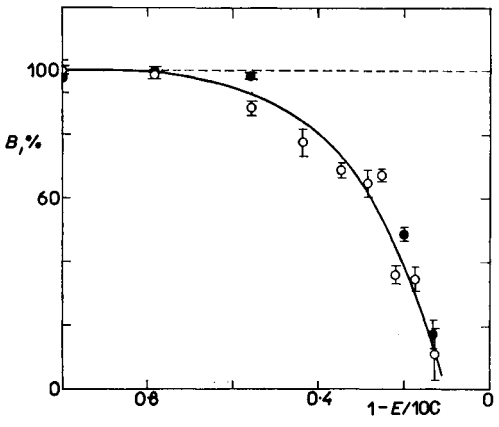

FIG. 2

Effect of the linear charge density of pectin on the formation of the complex with poly-(Lys-Ala-Ala). B The helix-forming efficacy in %; $1 - E/100$ the linear charge density (*cf.* the text); poly(Lys-Ala-Ala) sample *I* ●, sample *II* ○

of complexation relates to the maximal mean value $\Delta\varepsilon_{225}$ found with poly(Lys-Ala-Ala) samples *I* or *II* ($B = 100\%$). Scattering of values at various ratios of both components in the mixture is not systematic and is, therefore, presented by the average of five runs and its mean error.

This dependence documents the ability of poly(Lys-Ala-Ala) to enter the interaction with pectinates under formation of a helical structure. The helix-forming effect of the pectinate remains significant even at a considerable drop of the linear charge density of pectinate at an esterification degree E 56·3%. It is noteworthy that the effect of interaction of poly(lysine) with pectin of E 64% already totally collapsed[9]. This difference in the action of both polypeptides reflects that of their charge densities and proves the importance of complementarity of linear charge densities of both interacting polyions in the complex structure.

The spatial arrangement of the poly(lysine)–pectate complex was tried to deduce in our previous paper[9]. The above-mentioned pectinates induce the same α-helical structure with both the sequential poly(Lys-Ala-Ala) and the homopolymeric poly(lysine). It remains open, whether the proposed models of superstructures are actual even for the investigated complexes with poly(Lys-Ala-Ala). Certain considerations reported in[9] have to be, however, met. The polypeptide molecule forms a characteristic α-helical structure (3·66 units per one turn; the turn pitch $h = 0{\cdot}54$ nm). We suppose the side linear chains with the terminal ($—NH_3^{(+)}$) groups to be oriented in the peptidic $C_\alpha—N—C(O)$ group plane and to have a *zig-zag* conformation. This spatial arrangement afforded the greatest distance of the terminal ($—NH_3^{(+)}$) groups. An important presumption tells that the mutual interaction need not to take place at distinct points. Charges of ($—NH_3^{(+)}$) groups of the polypeptide form a diffuse superficial electric field. The polyanionic pectate molecule surrounds this structure as an oppositely charged conductor. It occupies the most advantageous conformation for the equivalent mutual saturation of charge. The linear charge density of the helical poly(Lys-Ala-Ala) structure is very close to that of the unesterified pectate (E 0%). The distance between the neighbouring ($—NH_3^{(+)}$) groups in the poly(Lys-Ala-Ala) molecule in the perpendicular projection on the helix axis is 0·443 nm, that between the neighbouring ($—COO^{(-)}$) groups of the pectate is 0·437 nm. In other words, a condensation of the pectate charges along the polypeptide helix is not necessary for mutual saturation of charges by winding its chain round the polypeptide core to form a superhelix as with the pectate–poly(lysine) complex. The macromolecule of an unesterified pectate is here stretched parallelly with the helix axis of poly(Lys-Ala-Ala). This arrangement faces, at the same time, the problem of a full saturation of the charge field of the polypeptide at its cylindrical surface. It is obvious that the complete saturation of charges by the stretched pectate polyanion is impossible. Residual unsaturated charges of the polypeptide at the remote side of the cylindre surface of the helix can bring about a decrease of the thermodynamic stability of the complex. On the other hand, it enables the electro-

static interaction for further polyanionic molecules. It is obvious that neither the pectin charges are here fully saturated. At an excess of the polypeptide an interaction of pectin with further molecules of this polycation can occur. Such a formation of aggregates including several molecules might be the reason for the opalescence observed.

The low-esterified pectinates are almost fully involved in formation of the complex (Fig. 2). Increase of the esterification degree of pectin (*i.e.* a decrease of the number of anionic groups in its macromolecule) evokes a situation requiring a condensation of charges of the pectin chain to compensate the charge densities. This can be achieved by two ways similarly as proposed for poly(lysine): by an appropriate winding the pectinate macromolecule around the helical core of poly(Lys-Ala-Ala), or by an interaction with several parallel chains of pectin with the polypeptide. The first possibility, *i.e.* the formation of superhelical structure of the pectinate means some change in torsion angles of the glycosidic bond. The required change for lower esterification degrees of pectin involving a very gentle winding is minute; this deformation has to be considered at higher esterification degrees, where also a greater compression of turns of superhelical structure is necessary. The distance of the superhelix turns d corresponding to the equivalence of charge of both complex components was calculated for single samples of pectin of various esterification degree E, making use of geometric parameters reported in our previous paper[9]. The molecule of the helical polypeptide is characterized by the cylinder radius 0·94 nm, the surface of which represents the ($-NH_3^{(+)}$) groups of lysine side chains. The pectin molecule is approximated by a cylinder of the radius 0·55 nm. The calculated distances of turns d of the superhelix are given at the abscissa in Fig. 3. The plot characterizes the dependence of the complexation effectiveness (B) on the distance of turns (d) of pectinate necessary for a full saturation of charges of the

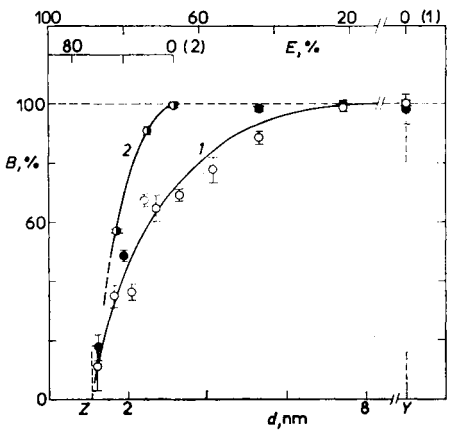

FIG. 3

Correlation between the complex-forming efficacy (B) of pectin of various esterification degree E and the distance of turns (d) of its macromolecule. *1* Complex with poly(Lys-Ala-Ala), *2* complex with poly(lysine). Point Y at the abscissa refers to structure of the complex without turns, point Z to the limit contact distance of turns

superhelix model. Curve *1* for the complexes with poly(Lys-Ala-Ala) shows that at an esterification degree of pectin $E > 56\%$ the distance between turns is great and therefore not enabling a perfect spatial saturation of the polypeptide charges. An increase in the complexation effectiveness at an excesss of poly(Lys-Ala-Ala) is here substantiated by formation of aggregates of several molecules. The compression of turns of the superhelix at $E > 56\%$ means an improvement of the spatial saturation of charges, but on the other hand, it brings about an already not negligible torsion of glycosidic bonds. At a greater approach of turns the electrostatic repulsive forces between single turns associated with carboxylate groups of pectin will come into effect. Fig. 3 shows for comparison purpose this dependence also for the complex with poly(lysine), curve *2*.

Both curves successively approximate with the increasing esterification degree of pectin (E) and limit to value Z ($d \sim 1{\cdot}1$ nm) characterizing the limit of the geometrical model of the superhelix and the direct contact of turns of the pectin macromolecule. Basing on the same point of zero complex-forming effect (Z) one can consider a similar spatial model of both polypeptide types. The necessity to saturate mutually and fully charges of both polyions found with poly(lysine) also holds for poly(Lys-Ala-Ala). The energetic stability of the complex at the same torsion of the glycosidic bonds is influenced by an unequal electrostatic effect, which consists of attraction of oppositely charged polyions, mutual repulsion of the pectinate turns, as well as of repulsion of terminal ($—NH_3^{(+)}$) groups. Results shown in Fig. 3 document this effect to be more favourable for formation of the poly(lysine) complex under assistance of pectin molecule with a three times higher linear charge density.

The second afore-mentioned alternative for the spatial arrangement is the interaction of the helical polypeptide with several parallel pectinate chains. Providing the equivalence of charges, the distance between turns remains identical as with only one interacting pectin chain. The complex containing two parallel chains of the superhelix can theoretically be considered with pectinate of esterification degree $E \geqq 50\%$, the complex with three chains for $E \geqq 67\%$, that with four chains for $E \geqq 75\%$, and that with five chains for $E \geqq 80\%$. As follows, the participation of several parallel pectinate chains is to be taken into account only with complexes, where the effect of interaction rapidly decreases. The question whether the estimated decrease of the complex-forming effectiveness is due to a distorsion of the mono-chain superhelical pectinate structure, or to a pure electrostatic repulsion associated with parallel chains of polyanions in the superstructure is hard to answer. Considering these experiments were carried out in very dilute solutions the winding of the polypeptide α-helix by the pectinate chain seems more probable than a simultaneous interaction of several chains.

The examination of models revealed that the dominant factor for the complex-forming interaction of acidic polysaccharides with peptides is the complementarity of charge densities at the surface of presumed conformations of both components.

This is exemplified by pectin of esterification degree $E = 65\%$, which induces in a predominant measure the helical structure of poly(Lys-Ala-Ala), but this ability is no more manifested with poly(lysine) of a high charge density. In favour of this statement is the fact that the α-helix of poly(lysine) was not formed on a contact with hyaluronic acid, desulfated chondroitin and keratin sulfate, having the linear charge density analogous with pectin of $E \sim 50\%$. The above-mentioned glycosaminoglycans do already exhibit the helix-forming interaction with poly(arginine), the helical structure of which has a greater radius and consequently, a lower superficial charge density. Poly(ornithine), having a higher superficial charge density, requires a polysaccharide with a high charge density for the helix-forming interaction. Really, of all hitherto investigated glycosaminoglycans (review[20]) this ability shows only heparin which is characterized by the highest content of sulfate and carboxyl groups in the macromolecule.

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