Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme

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Abstract. In this paper, the effects of pectin varying in overall charge (OCH) and local charge density (LCD) on the structure and thermal stability of Lysozyme (lys) are studied. At pH 5.1, the secondary and local tertiary structures of lys undergo a two-step change upon binding with increasing amounts of pectin. The fluorescence peak increases and shows a blue shift and the amount of helix conformation drops at a pectin/lys mass ratio (q) below or equal to that corresponding to maximal complexation (q_{max}). With increasing amount of pectin, the native like state is recovered at $q>q_{max}$. The decrease in helix content depends mainly on the OCH of pectin. Analysis of the thermodenaturation behavior of lys reveals that the affinity of pectin with denatured lys is higher than with the native state of lys. At the same OCH of pectin, unfolding of lys is more significant in case of a high LCD (blockwise distribution of charges) of the former. At $q=q_{max}$ (first stage of binding) the key factors stabilizing lys within its complexes with pectin against thermo-aggregation are a high LCH of pectin and, to a lesser degree, a low OCH. At $q>>q_{max}$ (second stage of binding), a high LCH of pectin is a key factor inhibiting thermo-aggregation of lys.

Keywords: *pectin- lysozyme, interaction, secondary structure, conformation, thermostability.*

1. Introduction

The importance of protein-polyelectrolyte (PE) interactions is obvious in modern biotechnological processes, for example, protein isolation [1-4], PE modification of protein substrates [5], immobilization of enzymes [6], and creation of functional nanobiomaterials [7]. Therefore, it is not surprising that the complexation of proteins with PE receives intense scientific interest, as indicated by the many studies in this area for various types of PE and formulation conditions [8-26]. Moreover, several reviews in this field are also available [27-29].

The binding of PE with proteins can result in different effects on protein thermal stability. Complexation can lead to an increase in thermal stability [30-32], or a decrease in thermal stability [21,25,33], or can have no effect on thermal stability [33]. Similar scenario's are possible for the secondary and local tertiary structure of a protein during and after its binding with a PE. The protein structure can undergo unfolding [24] or folding [33,34], or the structure can remain rather unaltered [35] depending on the structure of the ligand. Overall, the effects of protein-PE complexation on the protein structure and thermal stability are governed by a delicate balance between the different interaction forces that depend on several structural features of the protein and PE. This prompted us to investigate the influence of the local structural features of the ligand interacting with the protein on possible unfolding and folding processes and the thermal stability of the protein.

In the present work, the effects of the overall charge and local charge density of pectin on the structure and thermal denaturation and thermal aggregation of lysozyme (lys) were investigated. Circular dichroism and fluorescence spectroscopy were applied to assess the protein secondary

and tertiary structures during complexation with pectin, and DSC and turbidity measurements at various temperatures were applied to monitor the thermal stability of lys and its stability against thermo-aggregation. To our knowledge, such a combined application of complex structural methods sensitive to local changes of the protein structure, as well as its conformation and thermo-aggregation has not frequently been used in studies involving protein/polysaccharide systems.

Lys is a small (14.3 kg/mol) globular protein molecule with dimensions of 4.5 x 3.0 x 3.0 nm. The temperature of lys unfolding (T_m), and the thermal reversibility of this process depend on the physico-chemical factors of the solution, such as its pH and ionic strength [36,37]. The thermal stability of lys usually decreases during its interaction with anionic polyelectrolytes, such as heparin [38], dextran sulphate [23], kappa carrageenan [34], lambda carrageenan [39], polyvinyl sulfate [40,41], as well as polyacrylic and methacrylic acid [41].

2. Materials and methods

2.1. Materials

Highly purified Lys (>96% protein) was purchased from Sigma-Aldrich. Pectin with varying degree of methoxylation DM (varying overall charge density) and either a statistical (SP) or blockwise (BP) distribution of the methyl ester groups (varying local charge density) were produced as described in detail in earlier work [42]. BP pectin samples with DM values of 16.9% (BP16.9), 33.1% (BP33.1) and 68.2% (BP68.2), and SP pectin samples with DM 16.2% (SP16.2), 38.2% (SP38.2) and 66.5% (SP66.5) were used. For the preparation of buffers, milli-Q water and highly purified chemicals were used.

Solutions of biopolymers in an acetic acid-sodium acetate buffer (pH 5.1, I=0.01) were prepared as described in our recent work [42]. The protein concentration in the solutions was determined by UV absorption using an extinction coefficient of 2.64 ml mg^{-1} cm^{-1} at 281.5 nm [43]. The concentration of pectin in the solutions was determined by drying samples at 377K up to constant mass. If necessary, the pH values of lys and pectin solutions were corrected by 0.1–0.5M NaOH or HCl to remain at 5.1. All biopolymer solutions used in the experiments were previously kept in the refrigerator at 5°C for 12 h. to ensure complete hydration of the biopolymers.

2.2. Methods

2.2.1. Analysis of the complex phases

Analysis of the complex phases was carried out at different pectin/lys mass ratio (q) values. At first, the complex system was separated using centrifugation at 10000g for 20 min at 296K.Then,

the mass of the coexisting phases and the concentration of lys in the phases were measured. The concentration of lys in the diluted phase was determined by measuring the absorption value at 280 nm at high ionic strength (0.5 M NaCl) [34].The lys content in the complex phase was calculated by subtracting the amount of lys found in the supernatant from the total amount of lys in the complex mixture. The experimental errors were approximately 3%.

2.2.2. Analysis of the secondary structure

Analysis of the secondary structure of the lys solutions and pectin/lys mixtures was carried out using a Chiroscan Applied Photophysics spectrometer following the procedure described earlier [42]. A quartz cuvette with a light path length of 0.1 cm was filled with the sample solution. Circular dichroism spectra were recorded in the range of 195 to 250 nm with an interval of 0.2 nm. The solutions were scanned at a rate of 50 nm/min using a 2s time constant, a sensitivity of 20 mdeg and a step resolution of 0.1. The SOMCD method was applied to calculate the content of alpha helix, beta sheet and random structures on the basis of four scans for each solution [42,44].

2.2.3. Analysis of the local tertiary structure

Changes in the local tertiary structure during complexation were monitored by collection of the fluorescence emission spectra in the range of 300 to 450 nm. Spectra were recorded on a RF 5301 PC Spectrofluorimeter (Shimadzu, Japan) at 23°C with the excitation wavelength set to 280 nm, slit widths of 3 nm for both excitation and emission, and an integration time of 0.5s [42]. The fluorescence intensity was corrected for absorption of excitation light and re-absorption of the emitted light to decrease the inner filter effect [45]. The experimental errors did not exceed 2%.

2.2.4. Differential Scanning Microcalorimetry (DSC)

Effect of pectin on the thermodenaturation of lys was studied using a differential scanning calorimeter (DASM-4 M, Puschino, Russia) at pH 5.1. Thermograms were obtained between 10°C and 90°C, at a scan rate of 2K/min. Degassing during the calorimetric experiments was prevented by application of an additional constant pressure of 172.25 kPa over the liquids in the cells. The data were treated using WSCAL software Version 2.0 on the basis of the principles presented in [46]. The effective (van't Hoff) enthalpy of denaturation was calculated according to [47]:

$$\Delta H_{vH} = 4RT^2_m \Delta C_m / Q_m \quad (1)$$

where T_m is the thermal midpoint, ΔC_m the peak height (in JK^{-1}), and Q_m the area under the peak (in J).

3. Results

3.1 *Effect of overall charge and local charge density of pectin on the binding degree of lys in complexes*

First, the binding of lys in water insoluble complexes was estimated by means of separation of the coexisting phases and subsequent analysis of their composition. This allowed to determine the yield of lys (mass percentage) in the complex phase (Y_{lys}) (or the binding degree of lys in complexes with pectin) for systems containing pectin varying in structure. Figure 1a-c presents the yield of lys in the complex phase (Y_{lys}) for pectin/lys systems as a function of the pectin/lys mass ratio q. Figure 1 shows that for all the studied pectin samples, most of the lysozyme (≥78 wt%) binds in complexes with pectin regardless of the structural features of the latter. However, the amount of binding of lys to the complex with pectin increases with a decrease in the degree of pectin methoxylation (DM), that is, with an increase in the overall charge of pectin. In addition, the stoichiometric composition of the complex (pectin/lysozyme mass ratio at q_{Max}) is characterized by a higher content of pectin with a decrease in its overall charge (or increase of DM). Finally, the data suggest that the type of charge distribution along the pectin chain (statistical or blockwise) does not significantly affect the amount of lysozyme binding in the complex.

FIGURE 1

3.2. *Effect of overall charge and local charge density of pectin on the secondary and tertiary structure of lysozyme in complexes*

Secondary structure

Circular dichroism (CD) is frequently used to monitor changes in the chemical structure of proteins [50]. Therefore, this method was applied to determine possible effects of pectin on the stability of the secondary structure of lys. The CD spectra of pectin (data not presented) have a low intensity with a maximum at around 210 nm [51]. To collect accurate data on the change in secondary structure of lys in the presence of pectin, the CD spectra of pectin solutions with corresponding concentrations were subtracted from the spectra of lys/pectin mixtures. All measurements were carried out at 296K using a constant concentration of lys and an increasing mass ratio of pectin in the complex mixtures. Fig. 2a,b shows spectra of free lys and lys in the presence of SP16.2, which is highly charged pectin. The presence of a small amount SP16.2 in the lys solution (at q=0.1) leads to an appreciable decrease of the negative Cotton effect at 222 nm and 208 nm corresponding to a decrease of the α-helix content in lysozyme from 28.1% to 24.8%. The intensity of the spectrum at 222 nm further decreases with increasing pectin/lys mass ratio (q), and is minimal at q = 0.2 (Fig. 2a). Moreover, whereas the peak at 208 nm dominates in pure lys, the relative sizes of the peaks reverse at q = 0.2, which indicates structural changes of lys in the complex. At q=0.2,

the structure of lysozyme becomes almost unordered in the presence of SP16.2. It contains 8.2% α-helix structure, 10% β-sheets and 81.8% random structure. At q<0.3, the intensity of the negative bands decreases and at 200 nm an isodichroic point was observed (Fig. 2a), similar to studies on interactions of nanoparticles with proteins [53]. These results indicate that pectin/lys interactions modify the lys secondary structure (e.g. its α-helix content).

FIGURE 2

Contrary to the results at low q, increasing q up to 0.5 results in an increase in the amount of α-helix structure, and at q ≥0.5, the circular dichroism spectra become rather similar to that of the pure lys solution (27.4% α-helix, 9.0% β-sheets and 63.6% random structure) (Fig. 2b). The CD data obtained for the SP16.9/lys system at various q values allow to state that the decrease in the α-helix content of lys within the complex is a result of the formation of water insoluble complexes and their aggregation. At $q<q_{Max}$ increasing the q value leads to stronger aggregation and larger sizes of the complex particles [48,49] and simultaneously a lower α-helix content. On the contrary, at $q>q_{Max}$ increasing the q value results in strong disaggregation, and dissolution of the complex particles resulting in a lower content of protein in the concentrated phase (Fig. 1). Hence, under these conditions, the overall α-helix content of lys resembles more closely that of native lys.

Comparison of the CD spectra of the systems containing pectin with a statistical (Fig. 2a,b) and a blockwise distribution of the ester groups (Fig. 2c, d) at almost the same DM value and molecular mass of the pectin shows that the last system (BP16.9/lys) is characterized by a slightly larger decrease in lysozyme helicity at q values below and equal to maximum complexation. It should be noted that for both systems the yield of lys in the complex phase is similar. However, complexes of lys with pectin having a blockwise distribution of the ester groups are more aggregated [49]. This higher aggregation leads to a stronger decrease of the α-helix content in the BP16.9/lys system as compared to the SP16.2/lys system. Very similar CD data were obtained for SP38.2/lys and B33.1/lys systems (data not presented).

Figure 3a, b shows spectra of lys in the presence of different amounts of the weakly charged SP66.5 (Fig. 3a) and BP68.2 (Fig. 3b). Changes in the negative bands of the CD spectra in the presence of highly methoxylated pectin are similar but much less pronounced as compared to that in low methoxylated pectin/lys systems. This can partially be attributed to the reduced yield of lys in the complex phase for high methoxylated pectin. Moreover, the changes in the CD spectra are not sensitive to the distribution of the ester groups on the pectin chain, most probably because complexes from high methoxylated pectin are less aggregated as compared to low methoxylated pectin/lys complexes. More in particular, the α-helix content of SP66.5/lys systems decreases from

28.1% for free lys to 20.5% and 26% respectively at q = 0.3 and q=0.5, and to 23.1% and 29.8% for the BP68.2/lys system.

FIGURE 3

Based on the results for the secondary structure of the lysozyme protein in the presence of pectins it can be concluded that interactions between the oppositely charged macroions of lys and pectin, leading to the formation of pectin/lys complexes, and the resulting decrease in the surface charge of the protein and subsequent aggregation of the complex, cause a noticeable decrease in the helicity of lys in the complex as compared to that of free lys. Accordingly, an excess of pectin, leading to dissolution of the complexes, causes a partial (or complete) recovery of the secondary structure of lys. Therefore, the smaller the pectin overall charge (high DM pectin), the weaker the structural changes of the protein in the complex.

Tertiary structure

Tryptophan (Trp) fluorescence is frequently used in studies of the local tertiary structure of proteins [54]. Figure 4a-d shows changes in the fluorescence spectra of lys in the presence of highly charged pectins SP16.2 (Fig. 4a,b) and BP16.9 (Fig 4c,d) at various compositions. In the presence of SP16.2, with a statistical distribution of methyl ester groups, the fluorescence intensity of lys changed significantly (Fig. 4a). At $q < 0.3$ a significant increase of the fluorescence intensity of lys was observed (9.2% increase as compared to lys alone) up to q values approximately equal to q_{Max}. Moreover, a significant blue shift of the maximal emission wavelength from 335 to 332 nm can be observed (Fig. 4a). The blue shift of the fluorescence maximum of lys in the presence of SP16.2 is caused by the fact that the complex formation between pectin and lys places some of its Trp residues in a more hydrophobic environment. The observed increase in the fluorescence intensity of lys in the presence of SP16.2 is the result of insignificant conformation changes of the lys molecules. At $q>0.3$ the fluorescence spectra show an opposite trend with increasing q values, the higher q the lower the fluorescence intensity (Fig.4b). At q=0.8 the fluorescence intensity spectrum becomes almost the same as that of free lys. At the same time the wavelength corresponding to the maximum intensity shifts from 332 nm to 334 nm, i.e. closer to the value for pure lys. The changes of the fluorescence spectra of lys in the presence of BP16.9, with a blockwise distribution of methyl ester groups, are similar to that of SP16.2/lys systems, apart from two aspects (Fig. 4c, d). The maximum increase of the fluorescence intensity of lys in the BP16.9/lys system is much higher (24.8%) than that for the SP16.2/lys system, and its recovery at high q values (q=1.0) is only partial (Fig. 4d). Moreover, there is no appreciable shift of the fluorescence peak with increasing q.

The SP38.2/lys and BP33.1/lys systems, formed from pectins with intermediate charge density, present similar behavior of their fluorescence spectra (Fig. 5) as the SP16.2/lys and BP16.9/lys systems. The fluorescence intensity of lys in the presence of SP66.5 with low charge density changes insignificantly (1.5%) without a shift of the wavelength corresponding to maximum intensity (Fig. 6a). For the BP68.2/lys system the fluorescence intensity increases monotonically in the presence of pectin and the increase reaches 29% at q=1.0 (Fig. 6b). Thus, there is some analogy between the fluorescence and circular dichroism spectra of the pectin/lys systems with increasing q values. At $q<q_{Max}$ significant changes of the spectra are observed whereas at $q>q_{Max}$ when the yield of the complex phase decreases significantly, the spectra converge back to that of native lys. The more significant changes in the fluorescence intensity and CD spectra of lys in the presence of blocky pectin can point to more pronounced molecular rearrangements in these systems. Finally, the fluorescence spectroscopy data show that the tertiary structure of lys is stable in the presence of all pectins (there is no red shift). Moreover, the tertiary structure of lys in complexes with SP16.2 is even more stable (blue shift in spectra) than that of lys in complexes with BP16.9.

The results of the lys fluorescence analysis in the presence of pectin showed the presence of minor conformational changes in lys molecules, which are similar to changes in the secondary structure of the protein. In other words, the interaction of oppositely charged macroions of lys with pectin, which leads to the formation of an electrostatic complex, a decrease in the surface charge of the protein, and subsequent aggregation of the complex causes minor reversible conformational changes in lys. After the protein is recharged in the presence of excess pectin and the complex is dissolved, these conformational changes disappear.

FIGURE 4

FIGURE 5

FIGURE 6

3.3.Conformation stability of lysozyme under different thermal conditions.

The excess heat capacity, obtained from differential scanning calorimetry, for complex systems with SP pectins at different q values is presented in Figure 7a-c. The melting temperature (T_m) values and overall unfolding enthalpy (ΔH_{Cal}) at various DM values for pectin/lys mixtures at $q=q_{max}$ are shown in Figure 9a,b. Free lys shows T_m values of 350.5K (Fig. 9a) and an overall unfolding enthalpy of 568.57 $kJmol^{-1}$ or 39.76 Jg^{-1} (Fig. 9b), which is similar to the results of [38]. The presence of SP16.2 in the lys solution changes the parameters of thermal denaturation considerably (Fig. 6a, b). At q=0.05 the melting temperature and unfolding enthalpy decrease to

349.98K (Fig.7a) and 522.72 kJmol^{-1} or 36.55 Jg^{-1}(Fig.9b) respectively.In addition, in the second heating scan, the calorimetric melting peak is no longer visible, which shows the irreversibility of the heat denaturation process.

FIGURE 7

FIGURE 8

FIGURE 9

Increasing the pectin/lys mass ratio to q=0.21 (q_{max}) leads to a further decrease of the melting temperature T_m to 344.63K (insert in Fig. 7a and Fig. 9a) and unfolding enthalpy to 374.32 kJmol^{-1} or 31.8 Jg^{-1}(Fig. 9b). Further increasing q to 5.0 results in a further decrease of the unfolding enthalpy to 351.59 kJmol^{-1} or 24.59 Jg^{-1} (Fig. 9b) while the melting temperature remains the same (insert in Fig. 7a). The scans for complex SP38.2/lys systems at different q values are presented in Figure 7b. The data obtained are similar to those for the SP16.2/lys system. However, the maximal differences between the T_m values and unfolding enthalpy of lys alone and that in the presence of SP38.2 is less (4.52K and 152.42 kJmol^{-1}) as compared with those of SP16.2 (6.07K, and 217 kJmol^{-1}). The presence of SP66.5 in the lys solution does not lead to an appreciable decrease of the melting temperature and unfolding enthalpy of lys in the complete q range studied (from 0.05 to 5, Fig. 7c). It can thus be concluded that the thermo-aggregation behaviour of lys molecules is not affected by the presence of SP66. For the system containing blocky pectins (BP) more pronounced effects are observed (Figs. 8 and 9). The melting temperature T_m values are lower (338K-339K) as compared to those for systems comprising pectins with statistical distribution of methyl ester groups (SP) (349.3K-344.6) and the values depend only weakly on the DM value of the pectins. Moreover, the unfolding enthalpy is lower which indicates that lys is more prone to unfold in these systems. These more pronounced effects of blocky pectin on the thermal stability of lys support our observations of more pronounced molecular rearrangements in the presence of BP as compared to SP.

Thus, the study of the thermal stability of lys in the presence of pectin showed that the main factors determining the thermal stability of the protein in the complex are the overall and local charges of the pectin molecule. The higher the DM value of pectin with statistical distribution of methyl ester groups (i.e. the lower the charge) the higher the thermal stability of lys. Blocky pectins, which are characterized by a higher local charge density, reduce the thermal stability of the protein regardless of the degree of methoxylation (overall charge) of the molecule.

3.4. Effect of overall charge and local charge density of pectin on heat-induced aggregation of lysozyme

It is well known that many structural and conformation changes in proteins affect their aggregation under influence of different physico-chemical factors including temperature [55]. Therefore, it is logical to assume that pectin can affect the aggregation of lys. Figures 10 and 11 show the turbidity spectra in the visible wavelength range at different temperatures in the case of complex pectin/lys systems for two specific q values. In the first case (Fig. 10) the q values were equal to q_{Max}. In the second case an excess of pectin in the system was chosen as those complexes are mainly negatively charged and soluble (Fig. 11).

FIGURE 10

FIGURE 11

Thermal aggregation of pure lys in the buffer with pH 5.1 and I=0.01 starts at 328K (data not shown), whereby the extent of aggregation increases with temperature. The effect of adding SP with a statistical distribution of methyl ester groups is presented in Figs. 10a-c. The SP16.2/lys system undergoes aggregation which monotonously increases with temperature, already starting from 296K. From 343K onwards, the absorption values do not change anymore with increasing temperature (Fig. 10a). A similar thermal behaviour is observed for the SP38.2/lys system. The higher the temperature the stronger the aggregation. However, replacing SP38.2 by SP66.5 in the pectin/lys system results in the opposite behaviour (Fig. 10c). Moreover, the SP66.5/lys system is fully disaggregated when the temperature rises to 338K. Above 338K no further change of the turbidity values occurs (Fig. 10c). The thermal behaviour of the systems containing BP is quite different from that of systems containing SP. The former systems undergo very strong disaggregation upon heating; the turbidity values decrease 3-4 times even after limited heating from 296K to 323K, and they do not change with further increases in the temperature. Besides, the thermo-aggregation behaviour of the pectin/lys systems with blocky pectin (BP) does not depend on the DM of pectin (Fig. 10e-f). This qualitatively different thermal aggregation and disaggregation behaviour in the presence of either BP or SP indicates differences in the secondary interaction forces in the aggregation of the pectin/lys systems.

Figure 11a-f presents the thermo-aggregation behavior of the systems at q=5. It can be seen that at room temperature all systems studied have a very small turbidity due to solubilization of the complexes. The system containing SP has a slightly higher turbidity as compared with that containing BP, which decreases when increasing the temperature up to 50°C. Systems containing BP have a very small turbidity at 23°C that does not change with increasing temperature.

The results obtained allow to state that the main difference in the effect of pectins with either a statistical or a blockwise distribution of methyl ester groups on the thermal aggregation of lys in the complex systems is that the systems with blocky pectin (BP) always undergo thermal disaggregation whereas pectin SP causes thermo-aggregation at low DM and disaggregation at high DM.

4. DISCUSSION

The DM values and distribution of the ester groups on pectin determine its overall and local charge densities and also the extent of intermacromolecular hydrogen bonds during the interaction of pectin with lys. According to the phase analysis, the strength of the electrostatic interactions between lys and pectin weakens considerably starting from DM values of approximately 66%. The q_{max} value for the mixtures with BP68.2 is larger than that for the mixtures with SP66.5, although the maximal yield of lys in the complex phase was similar for both types of systems. The former peculiarity may be due to the stronger self-association of BP68.2 [56,57] which would result in less available sites for interactions with the lys protein.

Structural changes of lysozyme induced by pectin

The results obtained from the circular dichroism and fluorescence spectroscopy techniques allow to describe the structural changes of lysozyme induced by increasing amounts of pectin as a two-stage process, spanning a broad range of pectin/lys mass ratios (q). The results at low and high q respectively are summarized in Tables 1 and 2.

Stage 1 ($0<q<q_{max}$): The carboxyl groups of the pectin molecules interact with the positively charged groups of lys (Lysine, Arginine and Histidine residues), and the resulting reduction of the electrostatic repulsion between the complexes drives aggregation and subsequent precipitation after centrifugation. The data of the fluorescence spectroscopy of SP16.2/lys (Fig. 3a) and, to a lesser degree, BP16.9/lys systems (Fig. 3b) clearly show that after binding of the carboxyl groups of these pectin samples with lys, the free Trp residues become placed in a more hydrophobic environment (inside the globule) and undergo less solvent exposure [59]. Indeed, some Trp residues (Trp108 and Trp111) of lys have more motional freedom than the other Trp residues [58] and can thus undergo a transition into a more hydrophobic environment in the presence of SP16.2 and BP16.9.

An analysis of the CD spectra allows establishing the next interesting peculiarity: The degree of disruption of the α-helix structure of lys during its binding with pectin mainly depends on the overall charge of the pectin molecule, i.e. its DM value. The higher the overall charge of the pectin molecule the more pronounced the disruption of the lys α-helix structure. The binding of lys with

pectin results in a change of the α+β native structure of lys to the α/β structure, consisting of mixed segments of α-helix and β-sheet structures [59]. Since such structural changes take place for all systems containing SP, and are not observed for blockwise highly methoxylated pectin we can reliably assume that this change in the state of the β structure of lys during its binding with pectin requires a sufficient amount of charges but also a sufficient charge distribution along the chain.

Fluorescence analysis shows that the local tertiary structure of lys is sensitive to both the overall charge of pectin and its local charge density. The higher the content of charged carboxyl groups) (low DM) and their density along the pectin chain the more significant is the increase of the fluorescence intensity of lys. SP with a high esterification degree does not affect the tertiary structure of lys appreciably. In contrast to SP, BP modifies the tertiary structure of lys (without its unfolding) even at high DM values (68%). This structure change occurs in such a way that the protein is partly unfolded and loss of the helix content occurs but the overall native tertiary structure is maintained.

Stage 2 ($q>q_{max}$): Charge plays a significant role in this stage that includes the disaggregation and dissolution of insoluble pectin/lys complexes. The association with pectin enhances the overall negative charge of the complex, which increases the dissolution of the precipitates. Additional uptake of pectin to the complexes results in association of lys segments by reconstruction of the secondary structure with a high helix content [60]. Analysis of the CD spectra shows that the loss of the α-helix structure of lys during the first stage of the binding is compensated almost completely at $q>q_{max}$ probably due to dissolution of the complexes and the presence of free lys in solution instead of bonded one. However, the α+β structures of free lys determined by the ratio of the CD bands at 222 nm and 208 nm was only recovered for the system comprising BP. The system including SP keeps containing α/β segments in its secondary structure. Analysis of the fluorescence spectra clearly indicates that polysaccharides with a higher charge density and different DM values have effects on the local tertiary structure of lys, whereas SP affects the local tertiary structure of lys only at a high overall charge i.e. SP66.5 does not affect the tertiary structure of lys.

Conformation changes of lys within its complexes and stability against thermo-aggregation.

It can be concluded that almost all pectin samples (excluding SP66.5) reduce the thermal stability of lys. The fact that the binding with pectin decreases the melting temperature indicates that the affinity of pectin with denatured lys is higher than that with the native state of lys [61]. Similar observations have been made for systems containing lys and heparin [38], polyvinyl sulfate [38,40], polyacrylic and methacrylic acid [41]. Analysis of the DSC traces

and thermodynamic parameters of thermodenaturation of complex systems containing SP or BP with very similar DM value allows to conclude that the local charge density affects the thermal denaturation of lys more strongly than the overal charge of the pectin molecule. Finally, highly methoxylated SP66.5 does not affect the conformation stability of lys neither its local tertiary structure. Consequently, a low overall charge of carboxyl groups in the pectin molecule and a random character of their distribution along the chain are the key factors determining conformation stability and the stability of the protein to thermal denaturation in lys/pectin complexes. This can be attributed to the fact that the absence of charged blocks along the pectin chain reduces the electrostatic interactions between lys and pectin.

Analyses of the turbidity spectra of pectin/lys systems allows to draw the following conclusions:

a) Thermo-aggregation and disaggregation processes of lys in the presence of pectin occur ahead of the thermal denaturation process.
b) At $Y=Y_{max}$ (first stage of binding at $q \leq q_{max}$): The key factors promoting thermal disaggregation of lys within its complex are a high local charge of the pectin (BP16.9, BP33.1, BP68.2), and to a lesser extent, a low overall charge of the polysaccharide (SP66.5). Accordingly, the main structural factor initiating thermal aggregation of lys in the presence of a ligand is a high overall charge of the latter.
c) At $Y>>Y_{max}$ (second stage of binding at $q>q_{max}$): Thermal disaggregation of lys within its complexes takes place for the systems containing SP and it depends insignificantly on the overall charge of the polysaccharide. A high local charge density of the ligand is a key factor stabilizing lys within the complex against thermo-aggregation and thermo-disaggregation.

Conclusion

In conclusion, we demonstrate that the overall charge and charge density of pectin, as well as the pectin/lys mass ratio in mixtures determines the possible changes of the secondary and tertiary structures of lys within the complexes, and its thermal denaturation, and thermal aggregation/disaggregation equilibrium. Application of calorimetric and spectral techniques shows that structural changes of lys in the presence of increasing amounts of pectin can be described as a two-stage process, spanning a wide range of pectin/lys mass ratios (q). The combination of spectroscopic and calorimetric analysis techniques allows to unravel changes in the structure and the aggregation state of lys in the complexes. The results obtained are summarized in Tables 1 and 2. From the overview of all the characterization results, it can be concluded that there is not always a one-to-one correlation between the different properties of lys

in the complexes such as its secondary structure, conformation, thermal denaturation and thermal aggregation or disaggregation behaviour. This indicates that whereas some properties are more sensitive to the overall charge of pectin (DM value), others depend more on the local charge density (SP versus BP) or on a combination of both properties. This study demonstrates an effective experimental approach to study complexation for different types of protein/polyelectrolyte combinations.

Highlights

-At $q \leq q_{max}$ the tertiary and secondary structure of lys change with increasing amounts of pectin.

- The decrease in helix content of lys depends mainly on the overall charge of pectin.

- The change in content of β sheets is determined by both the overall charge and the charge density of pectin.

- At $q > q_{max}$ the native state of lys is recovered.

- At the same overall charge of pectin, unfolding of lys is more significant in case of a high local charge density of the former.

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Table 1. Overview of results at $q \leq q_{max}$

	DM value	Charge distribution	
		Statistical	**Blocky**
Change of ordered structure	**Low**	↓↓	↓↓↓
	High	↓	↓
Fluorescence intensity	**Low**	↑	↑↑
	High	=	↑↑
$\boldsymbol{\lambda_{max}}$	**Low**	↓	=
	High	=	=
Melting temp T_m	**Low**	↓	↓↓
	High	=	↓↓
Unfolding enthalpy	**Low**	↓	↓↓
	High	=	↓↓
Thermo-aggregation	**Low**	↑	↓
	High	↓	↓

485 **Table 2.** Overview of results at $q > q_{max}$

	DM value	Charge distribution	
		Statistical	**Blocky**
Change of ordered structure	**Low**	↑↑	↑↑↑
	High	↑	↑
Fluorescence intensity	**Low**	↓↓↓	↓
	High	=	↑
λ_{max}	**Low**	↓↓↓	↓
	High	↑↑	↓
Melting temp T_m	**Low**	=	↓↓↓
	High	=	↓↓↓
Unfolding enthalpy	**Low**	↓↓	↓↓↓
	High	=	↓↓↓
Thermo-aggregation	**Low**	↓	=
	High	=	=

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Captions to figures and Tables

Table 1 Overview of results at $q \leq q_{max}$

Table 2 Overview of results at $q > q_{max}$

Fig. 1 The yield of lys in the complex phase for pectin/lys systems as a function of pectin/lys mass ratio q for BP16.9/lys (a), BP33.1/lys (b), BP68.2/lys (c). Corresponding data for pectins with a statistical distribution of methyl ester groups are presented for comparison. pH 5.1, I=0.01, 296K, C^{M}_{tot} = 0.3 mass%

Fig. 2 Circular dichroism spectra for a lys solution and complex SP16.2/lys (a,b) or BP16.9/lys (c,d) mixtures at various pectin/lys mass ratios q. pH 5.1, I=0.01, 296K, C^{M}_{lys} = 0.005 mass%

Fig. 3 Circular dichroism spectra for a lys solution and complex SP66.5/lys (a) and BP68.2/lys (b) mixtures at various pectin/lys mass ratios q. pH 5.1, I=0.01, 296K, C^{M}_{lys} = 0.005 mass%

Fig. 4 Fluorescence emission spectra for a lys solution and complex SP16.2/lys (a,b) and BP16.9/lys (c,d) systems at various pectin/lys mass ratios q. λ_{exc} =280 nm, pH 5.1, I=0.01, 2296K, C^{M}_{lys} = 0.005 mass%. The inset in b provides the position of the fluorescence maximum in nm as a function of q

Fig. 5 Fluorescence emission spectra for a lys solution and complex SP38.2/lys (a,b) and BP33.1/lys (c,d) systems at various pectin/lys mass ratios q. λ_{exc} =280 nm, pH 5.1, I=0.01, 296K, C^{M}_{lys} = 0.005 mass%

Fig. 6 Fluorescence emission spectra for a lys solution and complex SP66.5/lys (a) and BP68.2/lys (b) systems at various pectin/lys mass ratios q. λ_{exc} =280 nm, pH 5.1, I=0.01, 296K C^{M}_{lys} = 0.005 mass%

Fig. 7 DSC curves showing the excess heat capacity of lys and complex SP16.2/lys (a), SP38.2/lys (b) and SP66.5/lys (c) mixtures at various pectin/lys mass ratios q. pH 5.1, I=0.01, 296K, C^{M}_{lys} = 0.125 mass%.

Fig. 8 DSC curves showing the excess heat capacity of lys, and complex BP16.9/lys (a), BP33.1/lys (b) and BP68.2/lys (c) mixtures at various pectin/lys mass ratios q. pH 5.1, I=0.01, 296K, C^{M}_{lys} = 0.125 mass%

Fig. 9 (a) Unfolding temperature T_m of pectin/lys systems as a function of DM obtained from DSC data at q_{max}, (b) Calorimetric unfolding enthalpy ΔH_{Cal} for pectin/lys systems as a function of DM obtained from DSC data at q_{max}. pH 5.1, I=0.01, C^{M}_{lys} = 0.125 mass%

Fig. 10 Turbidity spectra as a function of temperature for SP16.2/lys (a), SP38.2/lys (b), SP66.5/lys (c), BP16.9/lys (d), BP33.1/lys (e), and BP68.2/lys (f) systems at q_{max}, pH 5.1, I=0.01, C^{M}_{lys} = 0.005 mass%

Fig. 11. Turbidity spectra as a function of temperature for SP16.2/lys (a), SP38.2/lys (b), SP66.5/lys (c), BP16.9/lys (d), BP33.1/lys (e) and BP68.2/lys (f) systems at q=5.0, pH 5.1, *I*=0.01, C^{M}_{lys} = 0.005 mass%

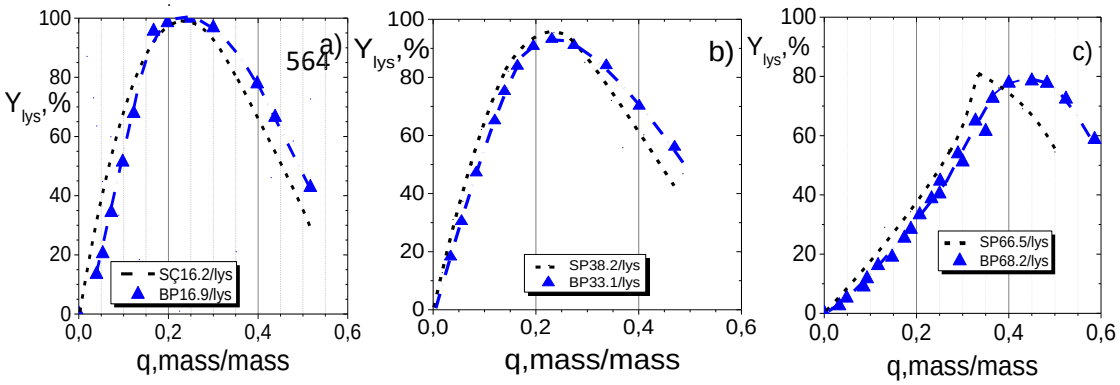

Figure 1. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

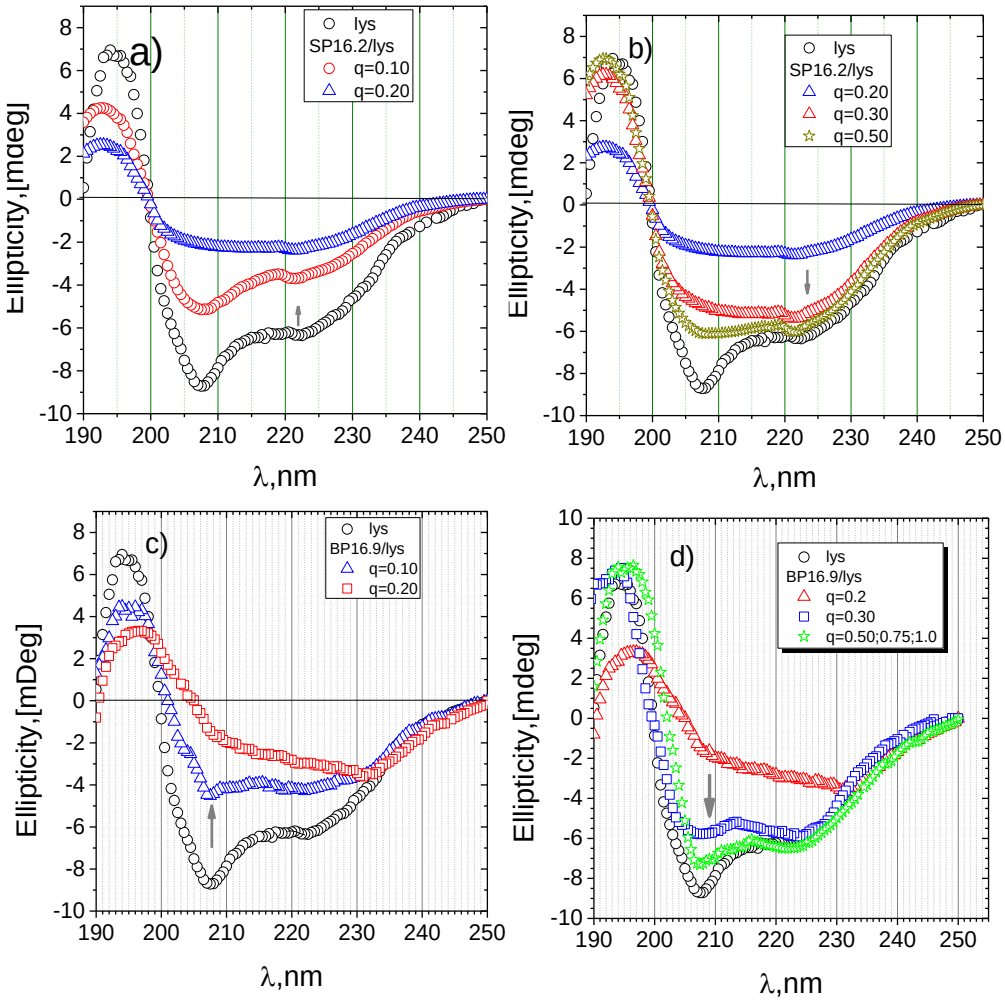

Figure 2. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

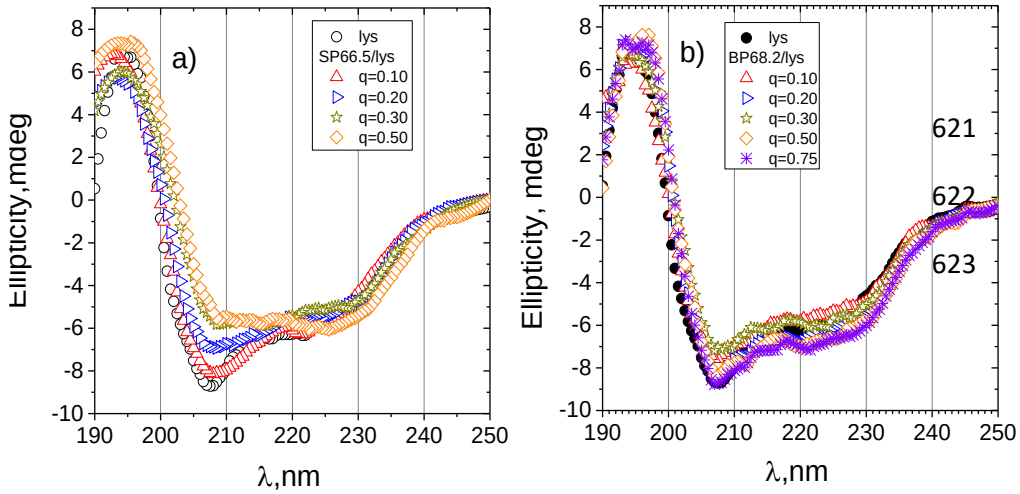

Figure 3. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

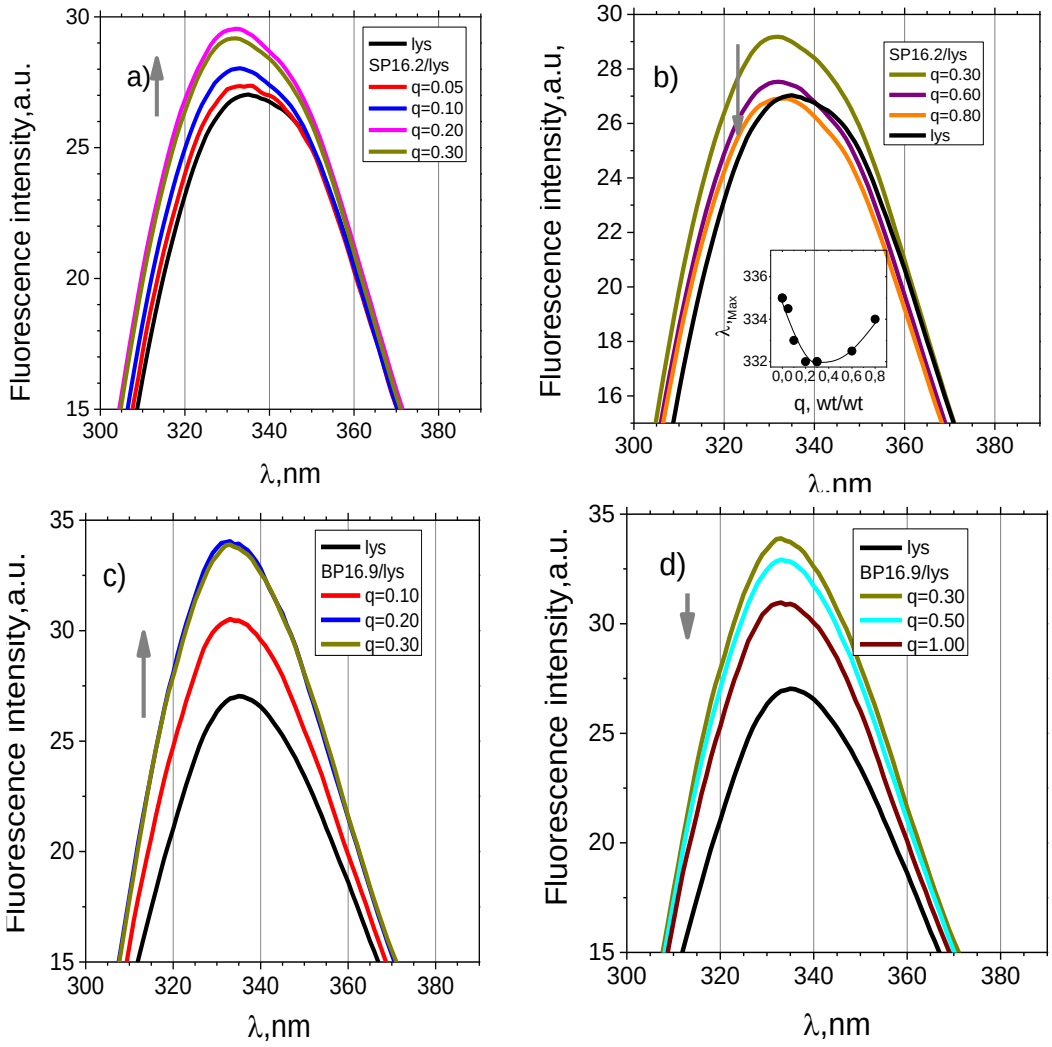

Figure 4. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

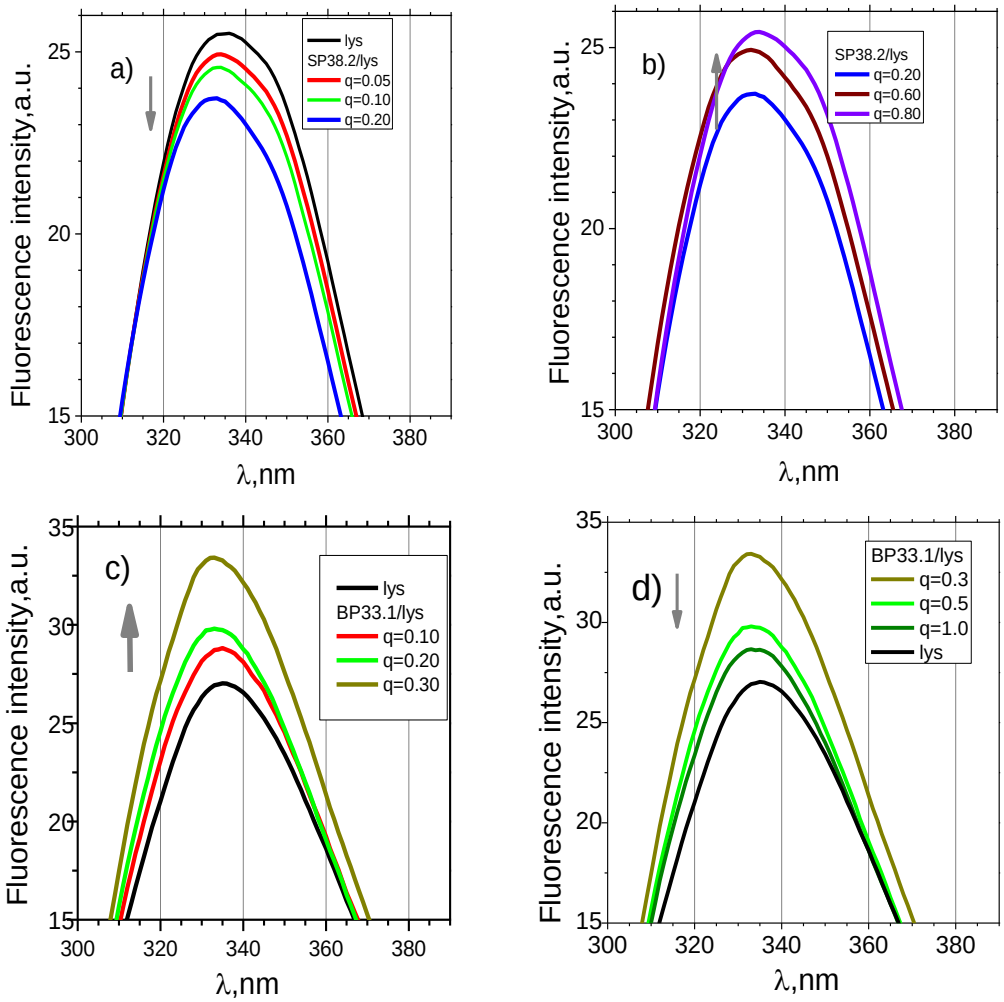

Figure 5. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

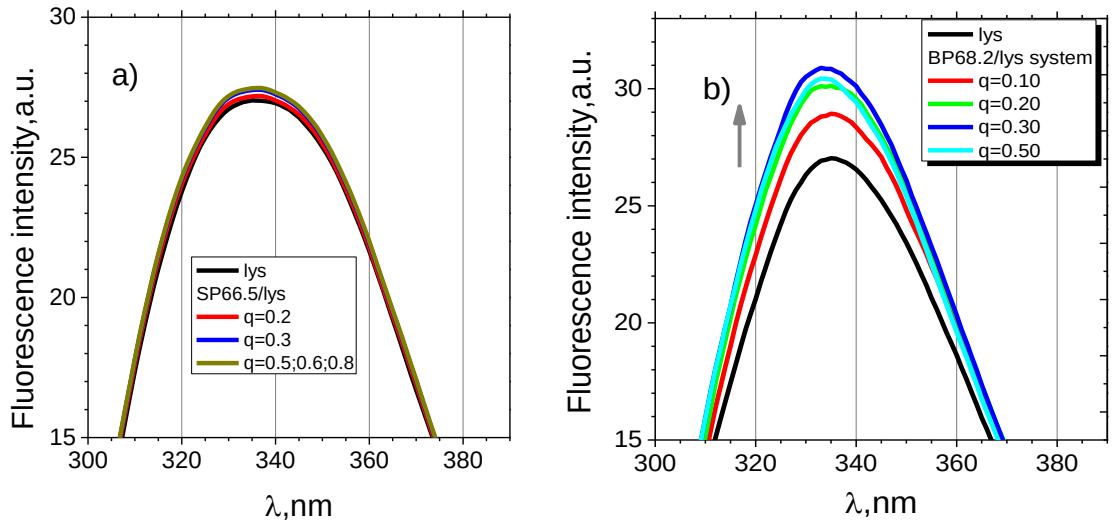

Figure 6. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

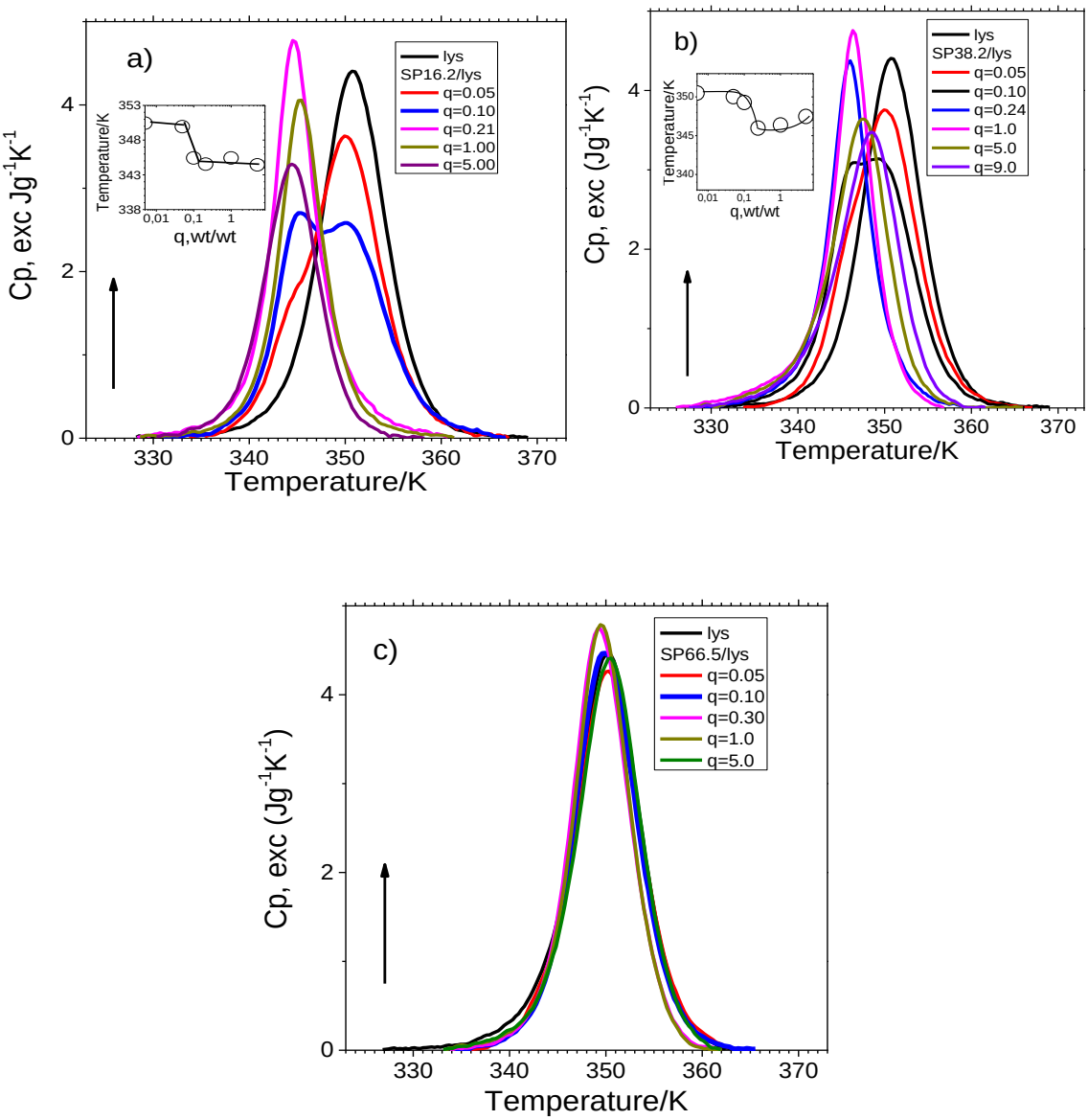

Figure 7. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

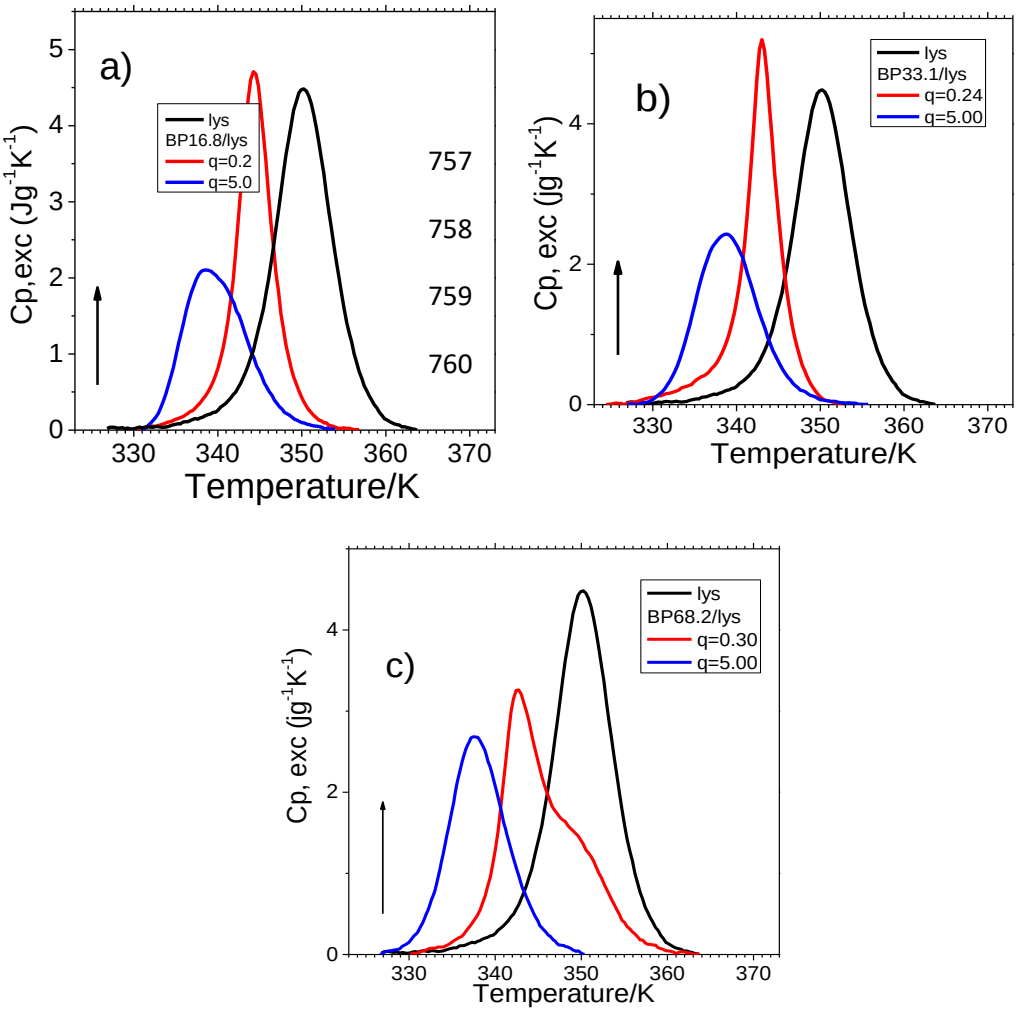

Figure 8. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

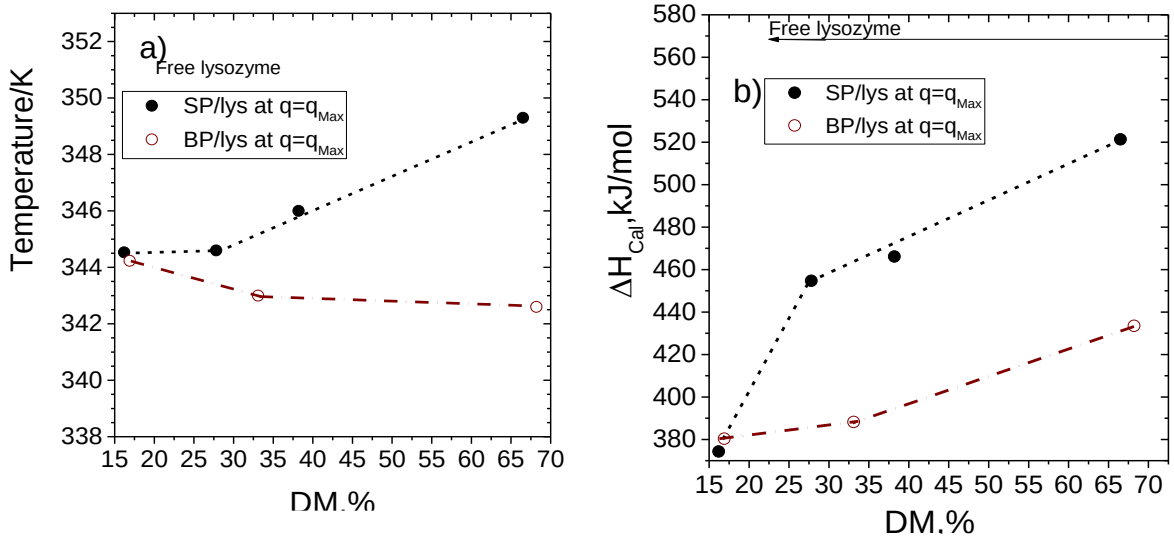

Figure 9. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

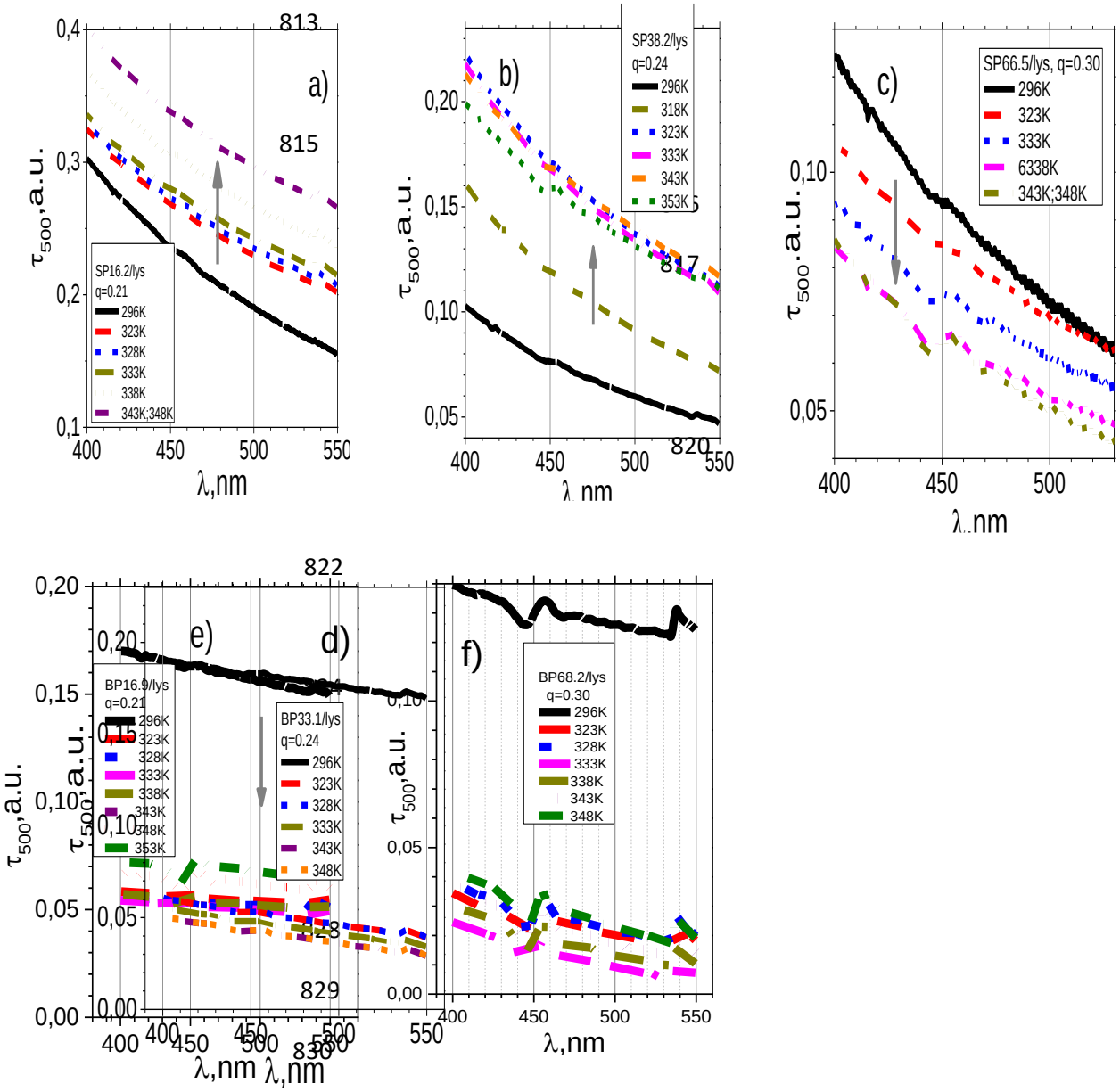

Figure 10. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

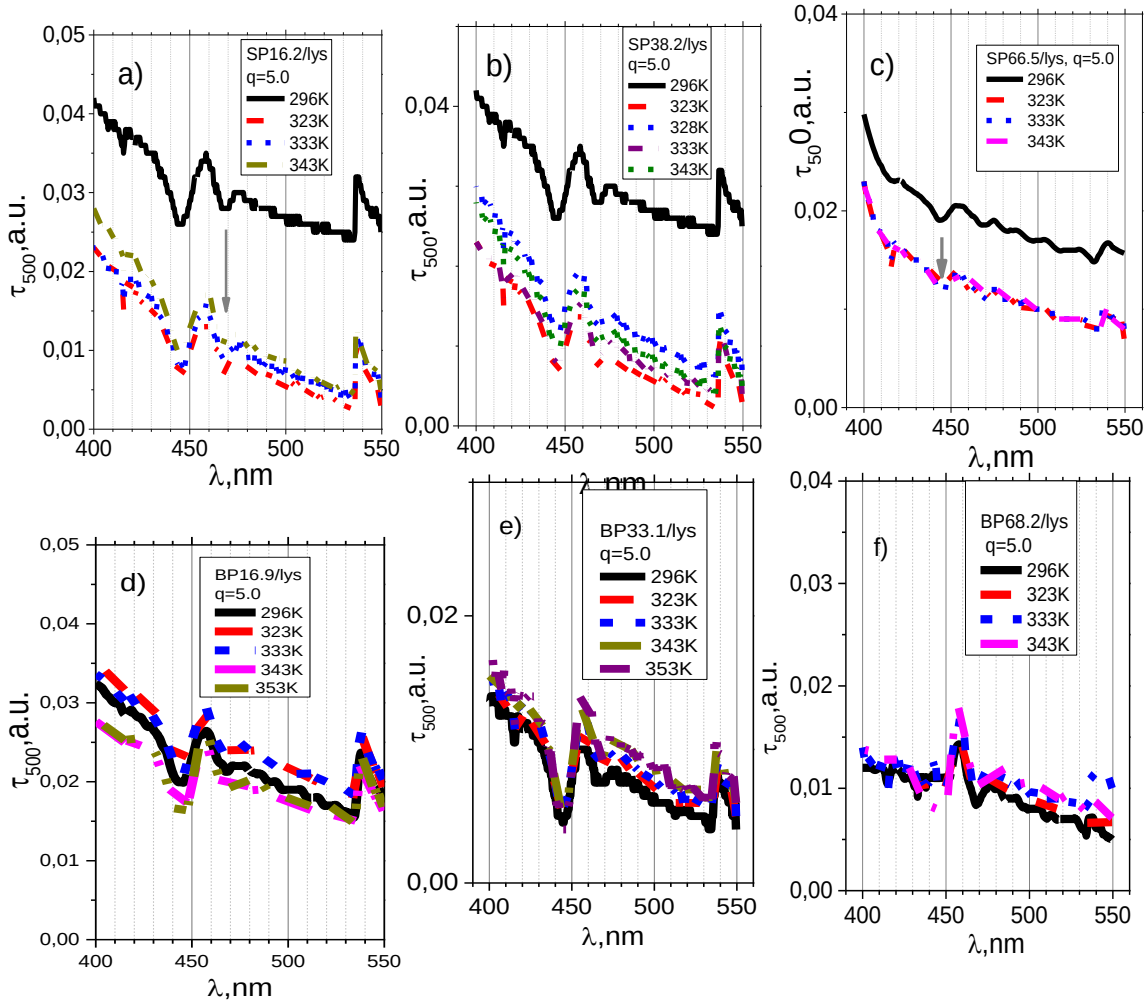

Figure 11. Y.A.Antonov et al "Effect of Overall Charge and Local Charge Density of Pectin on the Structure and Thermal Stability of Lysozyme"

Graphical abstract

