Conformational Modifications of α Gliadin and Globulin Proteins upon Complex Coacervates Formation with Gum Arabic as Studied by Raman Microspectroscopy

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As a molecular model of gelatin-free coacervates, complexes of pea globulin and α gliadin proteins with gum arabic prepared at different acidic pH values are studied using Raman microspectrometry. Raman spectra confirm higher content of β-sheets and random coils in pea globulin and dominating α-helical structures in α gliadin. For protein−gum arabic complexes, Raman data support the existence of specific pH conditions for optimal complex coacervation (pH 2.75 for globulin and pH 3.0 for gliadin[1]), when (i) pH-induced conformational perturbations of free protein structure are the strongest and (ii) compensation of these perturbations by gum arabic is the most pronounced. Conformations implied in the protein−gum complexes are mainly β-sheets in pea globulin and α-helix in α gliadin. The role of electrostatic and non-Coulombic interactions (intermolecular hydrogen bonds) in stabilizing of protein−polysaccharide complexes is discussed in relation with the overall structure and the charge density profile of these two proteins.

I. Introduction

Coacervation is a microencapsulation process used in pharmaceutical or food industries to protect sensitive substances such as vitamins or aroma, for example. The complex coacervation is commonly described as the simultaneous desolvation of oppositely charged polyelectrolytes leading to a dispersed phase rich in polymers and a continuous phase called the diluted phase.[2] The complexes are mainly formed and stabilized by means of electrostatic interactions between the amines and carboxylics groups borne by proteins and polyanions. These interactions are strongly dependent on pH, protein/polysaccharide ratio, and ionic strength in the coacervation processes. Recently, some complexes were shown to be less sensitive to pH and ionic strength. So, the non-Coulombic interactions were considered, with the involvement of NH and OH groups, as well as hydrophobic interactions shown by means of FTIR spectroscopy.[3] The physical interactions between macromolecules lead to hydrogen binding with, in a second step, conformational modifications which are dependent on the polymer−polymer and polymer−solvent interactions.[4] The ionic interactions are suspected to be stronger in the absence of hydrogen bonding, as suggested by the decrease of optimal pH value of complexation when urea is added to the mixture.[5]

Previous works showed the necessity of studying the structure of coacervation systems at molecular and macroscopic levels, since two oppositely charged polyelectrolytes do not lead systematically to coacervation. At a molecular level, it was shown using circular dichroism that complex coacervation between β-lactoglobulin and gum arabic induces a protein conformational change with a loss in α-helix,[6] but no general conclusion can be drawn from these results. It is necessary to implement this approach on a much higher number of systems under various experimental conditions, to sort out the general aspects from the system-specific ones.[7] The most classical system is a system where gelatin is used as the positive polyelectrolyte, and various polyanions such as gum arabic, alginate, pectin, and carboxymethylcellulose are used to counterbalance the positive charges of the protein. In the past years, due to the emergence of new diseases such as the prion desease, regulations concerning safety and health were reinforced. So, the research of new biopolymers which could be compatible with microencapsulation processes such as the coacervation process seems to be very important. Recently, complex coacervation was obtained with plant proteins as the cationic polyelectrolyte and gum arabic as the anionic one.[1] The effect of pH and protein/anionic compound ratio was investigated. The morphology and size of the coacervate particles according to these parameters were also studied by microscopic observations and laser granulometry measurements. Pea globulins fractions and α gliadins extracted from wheat were the main vegetal proteins used in this study.

Gum arabic (AG) is known as a hydrocolloid emulsifier, texturizer, and film-former. AG (E414, acacia gum) is a complex and variable mixture of arabinogalactan oligosaccharides and polysaccharides (M_w of major component is $\sim 0.25 \times 10^6$), as well as of hydroxyproline-rich glycoproteins (M_w of minor component is $\sim 2.5 \times 10^6$, ~2% protein).[8] Depending on the source, the glycan components contain a low or high content of L-arabinose, D-galactose, 4-*O*-methyl- and unsubstituted D-glucuronic acid, and L-rhamnose.[9] Since it is a mixture and the material

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varies significantly with the source, the exact molecular structures are still rather uncertain.

The pea globulin fraction isolated at pilot scale[10] was composed of legumin and vicilin. Both leguminous proteins are rich in aspartic and glutamic acids.[11] The vicilin is richer in lysin than legumin, and its isoelectric point is slightly higher (5.5 instead of 4.8 for legumin). The legumin is an hexameric protein whose molecular weight is 350 kDa, whereas the vicilin is trimeric with a molecular weight of ~150−180 kDa. Both proteins are β-structure-rich with a low content of α-helix (5−10%).[12]

The amino acid content and structural features of gliadins are different from those of globulins characteristics. Gliadins are seed storage proteins belonging to the prolamins group. These proteins are monomeric, and four types of gliadins (α, β, γ, and ω) can be distinguished on the basis of electrophoretic mobilities at acid pH in nondenaturing gels. In this study, the α gliadin is used. Its molecular weight determined by mass spectrometry is ~28−35 kDa.[13] The polypeptidic chain is organized in repetitive and nonrepetitive domains.[14] The N-terminal domain consists mainly of repeating sequences related to the sequence Pro-Phe-Pro-$(Gln)_{3-6}$, followed by a predominantly polyglutamine region.[15] The C-terminal domain includes six cysteine residues that form three intramolecular disulfide bonds, with a second short polyglutamine sequence.[16] The repetitive domain has a structure which is rich in β-turns, whereas the nonrepetitive domain is rich in α-helix.[17] The gliadins are poorly charged, and the majority of glutamic and aspartic residues are involved in amide functions. The isoelectric point of α gliadin is included in the pH range 6.5−8.0 (Autran, unpublished results).

Both frequency and intensity of molecular vibrations are sensitive to chemical changes and environment of the functional groups as reflected by changes in the Raman spectrum. Raman spectroscopy allows one to characterize structural features of complex polysaccharides, to determine primary and secondary structure of proteins,[18−20] as well as to study binding of proteins to other biomacromolecules.[21,22] The applicability of Raman spectroscopy to both solid and liquid samples makes it a useful tool for investigating protein structural changes in situ during aggregation, gelation,[23] or denaturation.[24,25]

The aim of the present study was to elucidate, using Raman microspectroscopy, major conformational changes of globulin and gliadin protein molecules responsible for formation of complex coacervates with gum arabic. Comparative study of the two structurally different proteins could provide insight into specific structural parameters important for protein−polyanion interaction. The use of laser scanning Raman microspectrometer in this study allows rapid measurements of numerous samples, each of them being of small size. An additional advantage of using the Raman mapping approach is that it provides a powerful analytical tool to rapidly and objectively treat series of spectra.

First of all, free molecules of gum arabic, globulin, and gliadin were characterized, both in commercial powder samples and after their solubilization in acidic pH ranging between 4.0 and 2.5. Then, the binary protein−polysaccharide mixtures were prepared at different acidic pHs. The small dehydrated fractions of the complex coacervates were analyzed using Raman microspectrometry with red excitation (632.8 nm). The spectra of complexes and of free molecules were interpreted in terms of primary and secondary structure of proteins, taking into account electrostatic interactions and conformational changes of gum arabic.

II. Materials and Methods

Chemicals. Pea globulin and α gliadin were the vegetal proteins used to be mixed with gum arabic. The pea globulin fraction was isolated at the pilot scale and α gliadin was purified using chromatography.[26] Gum arabic, which is extracted from acacia, was purchased from Cooper (France). Ultrapure water (Millipore system: Milli-Q Plus 185; Molsheim, France) was used to prepare the solutions. Acetic acid and sodium hydroxide were purchased from Riedel de Haën and were high-purity grades (>99%).

Sample Preparation. All the solutions were prepared at a concentration of 10 mg/mL. Plant proteins were dissolved in a 1% (v/v) acetic acid solution. The gum arabic preparation was solubilized at 40 °C under stirring (250 rpm until complete dissolution). The pH of each solution was adjusted to the desired pH with a 25% (v/v) acetic acid solution and a 0.1 M sodium hydroxide solution.

At three acidic pH (between 4.0 and 2.5) complex coacervates with a protein/polysaccharide ratio of 1:1 (w/w) were prepared at 30 °C by adding the polysaccharide solution (10 mg/mL) to protein solution (10 mg/mL) under stirring (500 rpm). The 20 mL batches were prepared in 50 mL beakers and the mixtures stirred at 500 rpm using a mechanical stirrer (3 blades of 2 cm diameter). After 1 min of mixing, a sample of the coacervate is taken out for analysis.

Raman Measurements. The Raman spectra were recorded on a LabRam (Jobin Yvon, Horiba) confocal scanning Raman microspectrometer equipped with an Olympus BX 41 microscope and an internal, air-cooled, helium−neon laser source providing radiation at 632.8 nm. The Raman scattering was excited and collected through a 100× objective (numerical aperture 0.90). The spectra were dispersed on a 1800′/mm diffraction grating and detected on a Peltier-cooled charge coupled device (1152 × 298 pixels). The spectral slit width corresponded to a resolution of 2 cm^{-1}.

The powder samples were analyzed directly, without any special preparation. The aqueous solutions were deposited onto a microscope glass slide and dried at ambient conditions (ca. 20 °C). No sample degradation was observed under the conditions used (5 mW laser power on the sample, 36 scans of 5 s).

A computer with LABSPEC 4.04 software was used for the data acquisition and treatment. The spectra presented were corrected for the fluorescence background with a spline function.

III. Results and Discussion

Raman Spectra of Gum Arabic. Raman spectra of gum arabic at different acidic pHs are shown in Figure 1. The spectra are dominated by vibrational bands of polysaccharide moieties; the presence of low-protein fraction (~2%) is revealed by a very weak and poorly resolved signal at wavenumbers higher than 1600 cm^{-1} (amide I region, described below). Approximate estimation of the protein/polysaccharide spectral fraction can be achieved from the intensity ratio of the amide I band over that at 1080 cm^{-1}. For all the samples of gum arabic, this ratio was ≤0.1. The pH decrease from 4.0 to 2.5 results in decrease of the bands at 1412 and 1344 cm^{-1} (COO^- and CH bending, respectively), increase of the bands at 1141 and 1028 cm^{-1} (C−O−C and C−OH stretching, mixed with C−C stretching), and increase of the bands at 877 and 839 cm^{-1} (C−C, C−O deformations, assignments according to literature[27,28]). The carboxyl protonation at the lowest pH gives rise to a carbonyl band appearing at a wavenumber higher than 1700 cm^{-1} (not shown). These changes are in agreement with protonation of the polysaccharide moiety that results in neutralization of carboxylates ($pK_a \approx 4.7$) and in conformational changes of glycosidic linkage. Powder of gum arabic was found to be in the acid form as evidenced by the corresponding spectrum (shown with dotted line in Figure 1).

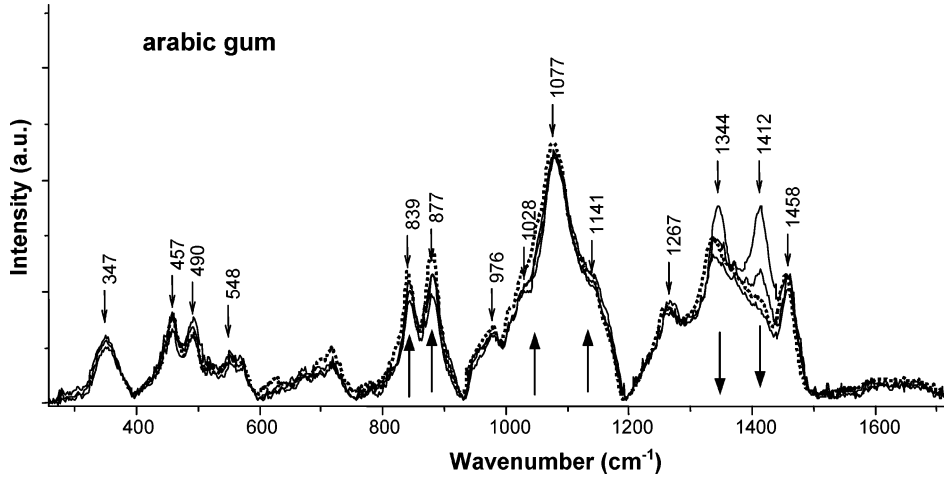

Figure 1. Changes in the Raman spectra of gum arabic upon pH decrease (indicated with arrows) below pH 4.0.

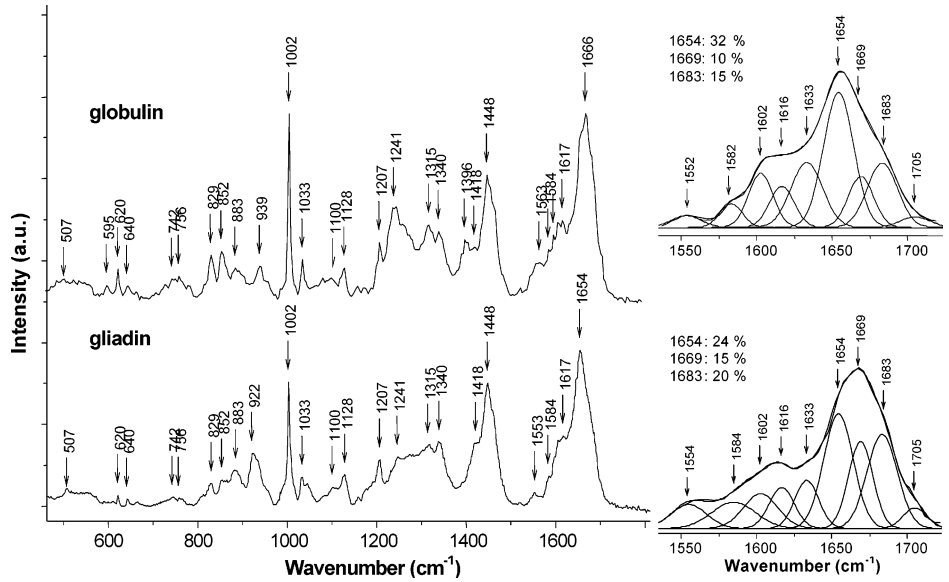

Figure 2. Typical Raman spectra of globulin and gliadin powder samples. For comparison, the spectra were normalized on the intensity of the 1448 cm^{-1} band. Insert: Gaussian-fitting of the respective amide I band, normalized to provide 100% overall area of the concerned region.

Spectral Features Related to Primary Structure of Globulin and α Gliadin. Raman spectra of globulin and α gliadin powder samples are shown in Figure 2. The frequency of the major bands and their tentative assignment, proposed in agreement with previous studies,[19,24,29] are resumed in Table 1.

The difference in the amino acid composition of gliadin and globulin is confirmed by several regions of the Raman spectra of these proteins. Concerning the bands of Phe aromatic amino acid, i.e., the most intense one at 1002 cm^{-1} (ring breathing mode) and the smaller ones at 620 and 1033 cm^{-1}, they show a higher intensity in the spectra of globulin than in that of gliadin powder. Nevertheless, the Phe band intensities were found to depend on the pH (more or less acid) of the samples extracted from solution. Therefore, their intensity is related not only to the Phe content, but also to the environment of this aromatic amino acid. The Tyr is responsible for the Raman bands at 1563 and 640 cm^{-1}, as well as with the Fermi resonance doublet at 852 and 829 cm^{-1}. These bands are somewhat stronger in the spectra of globulin compared to gliadin. The intensity ratio of the distinct vibrational bands at 620/640 cm^{-1}, indicative of the Phe/Tyr relative content, is higher in the globulin than in the gliadin spectra. On the other hand, the intensity ratio of the Tyr bands at 852/829 cm^{-1} is similar for both proteins. This ratio is known to be environmentally dependent and to increase upon tyrosine exposure to the polar medium or to interaction. The Trp amino acid content should be close in gliadin and globulin, since the characteristic Trp bands (756, 883, 1340, 1553, and 1584 cm^{-1}) have comparable intensities in the spectra of both proteins.[19]

Secondary Structure Features in Raman Spectra of Globulin and α Gliadin. The secondary structure of protein can be studied via Raman vibrational bands such as disulfide bridge stretching and the so-called amide bands assigned to the peptide bonds.[30]

The disulfide bridges are known to give stretching vibrations between 500 and 540 cm^{-1}, depending on the conformation of the C−C−S−S−C−C bonds in gauche−gauche−gauche (ca. 510 cm^{-1}) or trans−gauche−gauche (ca. 530 cm^{-1}). This information is difficult to obtain surely from the Raman spectra of both globulin and α gliadin, since the corresponding region contains only weak bands.

The regions situated near 1650 and 1250 cm^{-1} in the protein spectra are known as amide I and amide III regions, since they are mainly contributed from various vibrational modes of peptide or amide (−C═O−N−H−) bond. Both amide I and amide III

Table 1: Tentative Assignment of Characteristic Raman Bands of Gliadin and Globulin

band frequency cm^{-1} globulin (gliadin, if different)	tentative assignment vibration mode	primary structure	secondary structure
2927 (31) s[a]	C−H asymmetrical stretching		
~2874 sh	C−H symmetrical stretching		
1666 (54) s	C═O stretching		amide I
1683			β-sheet
1669			random coil and β-turn
1654			α-helix
1633			α-helix
1617 sh	ring stretch, side chains		
1602			
1584 w	ring stretch	Trp	
1562 (52) vw	ring stretch	Tyr (Trp)	
1448 m	$C-H_2$ scissoring		
~1418 sh	COO^- bending; $C-H_2$ wagging		
1340 m	$C-H_2$ bending	Trp	
1315 m	$C-H_2$ bending		
1241 m	N−H, C−H, and $C-H_2$ bending		amide III
1260−1270			α-helix
~1245			random coil
~1240			β-sheet
1207 m	C−C stretching		
1128 w	C−C stretching		
1100 vw	C−C stretching		
1033 w	C−C stretching	Phe	
1002 s	ring breathing	Phe	
939 (22)	C−C, skeletal stretching		α-helix
883 w	C−C, C−O deformations	Trp	
852 w	Fermi resonance	Tyr	
829 w	Fermi resonance	Tyr	
756 vw	C−C, C−O deformations	Trp	
640 vw	ring bending	Tyr	
620 vw	ring bending	Phe	
~507 vw	S−S stretching		g-g-g

[a] s, strong; m, medium; sh, shoulder; w, weak; vw, very weak.

regions are usually analyzed in order to interpret the composition and the secondary structure of proteins.

In the amide III region (1230−1320 cm^{-1}), the shorter frequencies are assigned to antiparallel β-sheet (ca. 1235 cm^{-1}) and to the random coils (ca. 1240 cm^{-1}), while the higher frequencies (ca. 1250−1270 cm^{-1}) are attributed to α-helix.[19,24]

In the amide I region of the Raman spectra of gliadin and globulin, the derivative spectra indicate the position of major components assignable to the α-helix, random coil, and β-sheet conformations at 1654, 1666, and 1684 cm^{-1}, respectively.[31]

The short-frequency position of the amide I band maximum for gliadin powder (1654 cm^{-1}, Figure 2) indicates the dominant presence of α-helices. According to literature, the α-helical structures of gliadin are likely to be mainly located in the C-terminal domain.[16] In contrast to gliadin, globulin has the amide I maximum at 1666 cm^{-1} that indicates β-strands and random coils to be the major secondary structures of this protein.[19,25] The higher random coil/α-helix ratio in globulin is also observed from the higher intensity ratio of the 1240/1270 cm^{-1} bands in the amide III region. The intensity in the region of the skeletal C−C stretching, near 930 cm^{-1}, is lower in the globulin spectrum (922 cm^{-1} for gliadin and 939 cm^{-1} for globulin) and could also be related to the lower presence of α-helices.[16,32] These results are in agreement with literature where plant globulins are described as relatively poor in α-helical structure.[25,33]

Quantitative estimation of the protein secondary structure is usually obtained via fitting the amide I band with theoretical (Gaussian or Lorentzian) profiles. The absolute quantification requires Raman data to be corrected for the water bending vibration at 1650 cm^{-1}, calibrated with additional data (for instance, those from infrared absorption and circular dichroism), and was not a goal in our study. Knowing the relative spectral contribution of the α-helix, β-sheet, and unordered structures is enough to monitor comparatively the conformational changes for the proteins upon complex coacervation.

Maiti et al.[34] have recently proposed a model system using three structurally relevant Raman markers in the amide I band. A simple, three-component amide I contour fitting was sufficient to explain the spectra of other natively unfolded proteins, namely, phosvitin, α-casein, and β-casein, and provided reasonable estimates of their preferred secondary structure. In our study, we found that three components centered at 1654, 1669, and 1685 cm^{-1} (positions determined from derivative spectra), and assigned respectively to α-helix, random coil (probably complemented with β-turn), and β-sheet, proved sufficient to account for the spectra of both proteins and their changes upon complex coacervation at different pH values.

For the proteins in powder, the spectral contribution of the respective Gaussian components expresses the quantitative difference between amide I regions of gliadin and globulin Raman spectra (insert in Figure 2, Figure 4). The contribution of the component at 1654 cm^{-1} (α-helix) is higher in α gliadin (~32% of the overall amide I band area) than in globulin (~24%). Inversely, the contributions of both β-sheet (component at 1683 cm^{-1}) and random coil (component at 1669 cm^{-1}) are lower in gliadin (15% and 10%, respectively) than in globulin (20% and 15%, respectively). To summarize, the spectral fraction of α-helical conformation is dominant in gliadin (32% > 25% (= 10% + 15%)), while in globulin, it is compensated by higher content of β-strand and random structures (24% < 35% (= 20% + 15%)).

Effect of pH on the Secondary Structure of Globulin and α Gliadin. The spectral changes observed on going from initial (powder) protein samples to those extracted from acidic aqueous solutions are shown in Figure 3. The pH-related changes of the main components of the amide I band are detailed in Figure 4 (see the empty symbols for the proteins alone).

In the amide I spectral region of both proteins, the pH reduction leads to the overall intensity loss (Figure 3) and to the increase of the higher-frequency components (Figure 4). These are in agreement with the expected reduction of α-helical fraction. The random coil spectral fraction is increased in the amide I region of both proteins. Interestingly, the highest content of disordered structure is achieved at pH 3.0 for α gliadin and only at pH value of 2.75 for globulin. The behavior of β-sheet spectral fraction in this region is remarkably different. In globulin, it remains nearly unchanged up to pH 3.5 and then is reduced at the most acidic pH 2.75. In gliadin, the β-fraction increases significantly up to pH 3.0 and then is slightly reduced. For this protein, the β-fraction remains much higher than that

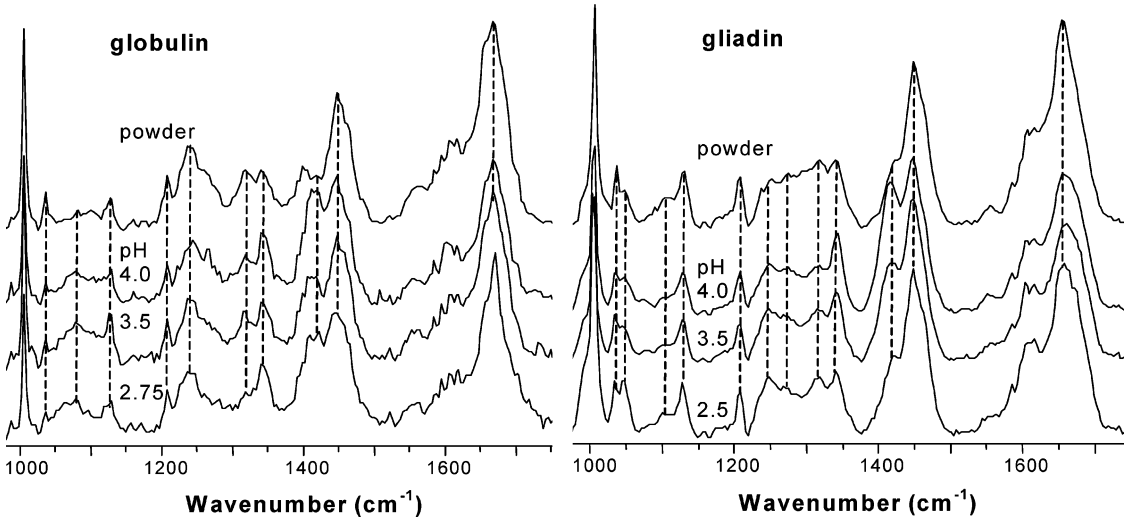

Figure 3. Major changes in the Raman spectra of free proteins, globulin, and gliadin on going from powder to acidic pH. For comparison, the spectra were normalized on the 1448 cm^{-1} band.

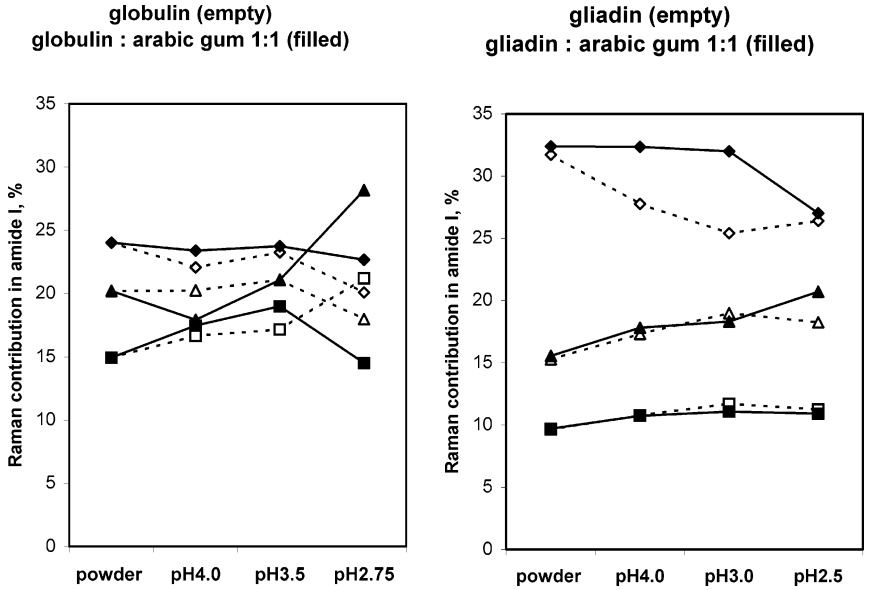

Figure 4. Relative contributions of three major conformation-related components (Gaussian-fitting made as for Figure 2) in the amide I band of free proteins (empty) or in the presence of gum arabic, 1:1 mass ratio (filled): ◇ and ◆, 1654 cm^{-1} (α-helix); □ and ■, 1669 cm^{-1} (random coil together with β-turn); △ and ▲, 1683 cm^{-1} (β-sheet).

of random coil over all the pH values studied, while for globulin, it is dominated by random coil at extreme pH. These results indicate that, at the acidic pH, α-helix is preferentially converted into random coil for globulin and into both random coils and β-strands for gliadin. At the most acidic pH, the described data from amide I of both proteins are indicative of protein denaturation.

Data from the conformation-sensitive amide III region are complementary to the above conclusions. The relative decrease of the α-helix component (1270 cm^{-1}) is already observed in the gliadin spectra at pH 4.0, while in those of globulin, it is evidenced only at pH 2.75.

The protein protonation on going to more and more acidic pH is also confirmed by progressive decrease of the intensity ratio of the doublets at 1418/1448 cm^{-1} and 1340/1315 cm^{-1}, observed for both proteins. On the other hand, the intensity ratio of these bands is increased on going from powder to the sample extracted from solution at pH 4.0.

Both solvation and denaturation affect exposure of Phe, as indicated by the Phe bands at 1033 and 1002 cm^{-1}. For the extracts from pH 4.0, these were higher than for the respective powder samples. At the most acidic pH, they decreased for globulin and increased for α gliadin.

Taken together, all these results indicate protein denaturation when leaving the isoelectric pH range. At pHs far from the isoelectric point, side-chain repulsion of induced net charges leads to a partial protein unfolding, rupture of hydrogen bonds and a breakup of hydrophobic interactions.

In view of the above-mentioned observations and the shape of the curves in Figure 4, one can notice that pH 3.5 for globulin and pH 3.0 for α gliadin represent a certain critical pH value, prior to strong protein denaturation.

Conformational Changes in Complex Protein−Gum Arabic. The Raman spectra of globulin and α gliadin in the presence of gum arabic (1:1 w/w ratio) at acidic pH are shown in Figure 5. Logically, these spectra are mixtures of the bands of both polysaccharide and respective protein. Preparations with globulin contained significant spectral contribution of polysaccharide; the intensity ratio of the amide I band over that at 1080 cm^{-1} was between 1.1 and 1.2, without any clear relationship to pH.

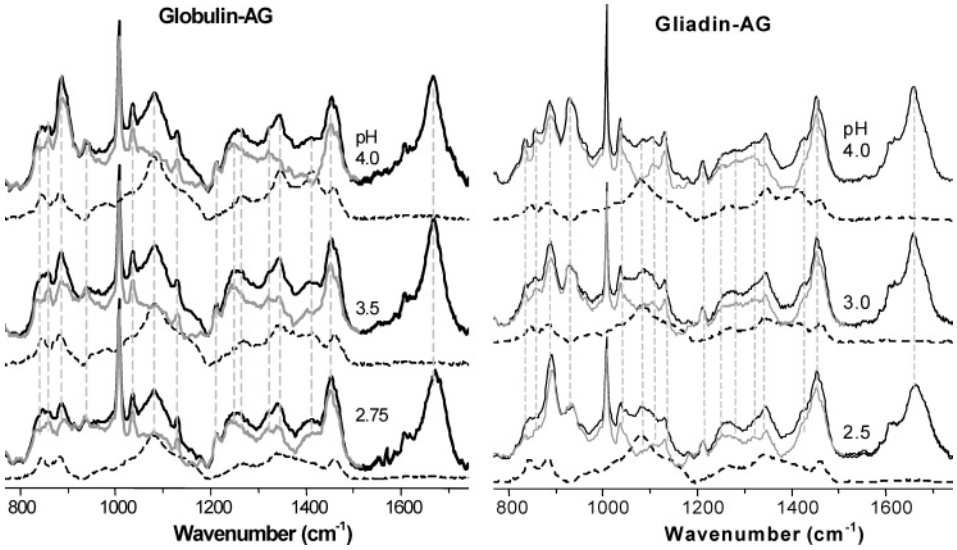

Figure 5. Major changes in the Raman spectra of globulin (left pannel) and gliadin (right pannel) in the presence of gum arabic (1:1 mass ratio) at acidic pH. Black solid: spectrum of the protein−gum complexes (dehydrated). Black dashed: spectrum of free gum arabic at the same pH. Gray: difference spectrum after subtraction of the gum arabic contribution from the spectrum of complex. For comparison, the spectra of complex were normalized on the 1448 cm^{-1} band.

For α gliadin complexes with gum arabic, this ratio was 2.1 at pH 4.0, 1.6 at pH 3.0, and 1.4 at pH 2.5. These indicate that α gliadin miscibility with gum arabic was systematically lower, although it increased at extremely low pH.

After subtraction of the contribution of free gum arabic from the spectra of complexes, the difference spectra (gray curves in Figure 5) point out the remaining structural modifications for both protein and polysaccharide components.

It is worthwhile noticing that in Figure 5 the amide I band seems not significantly affected by subtraction of the gum arabic spectrum, because of negligible protein signal of gum arabic (see Figure 1). On the basis of this observation, the Gaussian fitting of this band can still be realized in the spectra of protein−polysaccharide complexes (filled symbols in Figure 4).

Figure 5 provides a comparison of the conformation-specific features in the amide I band in the spectra of protein−polysaccharide complexes and free proteins. There are noticeable differences between complexes of globulin and α gliadin.

For globulin, the conformational perturbations (difference between filled and empty symbols) achieve the most important values at pH 2.75 (see the left panel in Figure 4). At pH 4.0, the perturbation concerns some decrease of the Raman band at 1683 cm^{-1} (*β*-sheet) and slight increases of those at 1654 and 1669 cm^{-1} (respectively, α-helix and random coil possibly contributed from *β*-turn). At pH 3.5, the bands at 1554 and 1669 cm^{-1} still increased, while that of *β*-sheet has the same intensity as for free globulin. At the lowest pH of 2.75, all the conformational Raman features are strongly affected by the presence of gum arabic. In comparison to free globulin at pH 2.75, the bands of α-helix and *β*-sheet are strongly increased (in particular the latter one), while that of random coil appears to decrease. To summarize, the amide I band analysis reveals that, at pH 4.0−3.5, the complex of globulin with gum arabic is favorable to α-helix and random coil conformations: The former is maintained as in the powder sample; the latter is slightly increased. At pH 2.75, the random coil increase in globulin is suppressed in favor of *β*-sheet.

For α gliadin at moderately acidic pH (4.0−3.0; see the right panel in Figure 4), the presence of gum arabic is also favorable to α-helical structures that are also maintained at a level close to that observed for α gliadin powder. In comparison to free α gliadin, the most important increase of the corresponding Raman feature at 1654 cm^{-1} is achieved at pH 3.0. Further pH lowering to a value of 2.5 completely suppresses stabilization of the α-helical component, that falls down to the level of free gliadin at the same pH. The *β*-sheet Raman feature at 1683 cm^{-1} remains unchanged down to pH 3.0, then somewhat increased at pH 2.5. The component at 1669 cm^{-1} of α gliadin appears insensitive to the presence of gum arabic at all pH values studied here.

Apart from the amide I band, the rest of the Raman spectra also contain significant structurally relevant information. Unfortunately, this information is hardly exploitable in the regions of strong spectral interference between bands of protein and polysaccharide. Although partially reduced after subtraction of the free gum arabic spectra, the interferences should be kept in mind while analyzing the complex spectra. For these reasons, we will discuss in detail only the most pronounced spectral features that differentiate the spectra of complexes (see the gray curves in Figure 5) from those of free proteins at the same pH (Figure 3).

For both proteins, the bands of COO^- and CH_2 deformational modes situated at ca. 1420 and 1340 cm^{-1} seem inhibited in the spectra of complexes, even if we consider the initial spectra before subtraction of gum arabic. The band at ca. 1420 cm^{-1} is particularly reduced at pH 4.0, that is concomitant with stronger implication of the negatively charged carboxylate functions (polysaccharide) in the electrostatic interaction with positively charged functions (protein). Reduction of the band at 1340 cm^{-1} is also in agreement with protein−polysaccharide molecular contacts, less obvious to interpret with more details.

The amide III region of complexes does not show any interpretable changes, as well as that of the lower frequencies between 1200 and 1000 cm^{-1} and below 800 cm^{-1}. In contrast, interesting Raman features are observed between 800 and 940 cm^{-1}.

One of the most noticeable changes is a strong increase of the band at ca. 880 cm^{-1} in the spectra of protein−gum complexes of both globulin and gliadin. For globulin, the increase is most spectacular at pH 4.0 and is progressively lost when going to lower pH. On the contrary, for gliadin, the increase is strongest at the lowest pH (2.5) and more moderate,

but still present, at higher pH. According to its position, this band could originate from C−C and C−O deformations of both polysaccharide and protein moieties (see assignments described above). The high-intensity rise of this band indicates rather strong conformational change in the related molecule. The flexible polysaccharide molecule should be strongly deformed upon interaction with proteins. From this point of view, it seems more reasonable to assign the band at 880 cm^{-1} preferentially to polysaccharide molecule vibrations. After subtraction of the free polysaccharide signal, this band is still very intense, indicating that gum arabic in complex with proteins leads to an increased intensity ratio of the doublet at ca. 880/840 cm^{-1}. This seems to be the major spectral feature related to the conformational adaptation of gum arabic in the presence of the protein molecule.

On the other hand, in proteins, the band close to 880 cm^{-1} is assigned to Trp amino acid. With the Trp contribution, the previously cited behavior could be, at least in part, related also to Trp environment changes in the presence of gum arabic. In contrast, no changes of Tyr environment could be documented, since the intensity ratio in the 852/829 cm^{-1} doublet is nearly unchanged in all the complexes.

Another interesting feature is particularly observed in the spectra of α gliadin−gum arabic complexes: The increase of the band at ca. 920 cm^{-1} is strong at pH 4.0 and disappears upon pH reduction. This band can be assigned to skeletal stretching in α-helical regions particularly rich in α gliadin. The comportment of this band confirmed our suggestion from amide I band analysis (Figure 4) that complexation with gum arabic favors α-helical conformation, particularly in gliadin. In a more general manner, the spectral changes involve only the intensities and not the frequencies of the bands, as expected for ionic protein−gum interactions.

Discussion of Protein Conformation in Relation to Optimal Coacervation. For pea globulin, the optimal pH for coacervation with gum arabic is 2.75,[1] since it allows one to obtain aggregates with good phase separation and a more regular shape than at higher pH. It is noteworthy that the coacervation of pea globulin at pH 2.75 was even more optimal after adjusting the protein−polysaccharide ratio from 50:50 to 30:70. The Raman data corresponding to complexes with a 30:70 ratio are not shown, since they were qualitatively similar to those with a 50:50 ratio.

For the free pea globulin, which is a protein rich in *β*-sheet, pH 2.75 corresponds to the decrease of *β*-sheet fraction. The *β*-sheet structure is characterized by a relatively large surface area that presents opportunities for ordered hydrogen bonds. Therefore, for free pea globulin at pH 2.75, the loss in *β*-sheet fractions indicates a loss of hydrogen bonds. The resulting unfolding of globulin is related to the decrease of α-helix relative fraction and increase of random coils. This unfolded conformation is favorable to interactions with the polysaccharide, since they are strongest at pH 2.75. Interestingly, within the complex globulin−gum arabic at pH 2.75, the protein is less disordered and the *β*-sheet conformation is particularly favored. The spectral contribution of *β*-sheet in the amide I region (Figure 4) is nearly 1.5-fold greater in the complex protein−gum than in the free protein at the same pH (2.75). These results suggest that the *β*-sheet domains are the most important structures for globulin−gum interaction.

For α gliadin, good phase separation with regular coacervate droplike particles was obtained at pH 3.0. At this pH, we observe the strongest loss of α-helical fraction for free gliadin and the strongest stabilization of α-helical structure in the protein−gum complex (the respective spectral contribution is ∼1.3-fold higher than in the spectra of the free protein at the same pH). In contrast to globulin, the *β*-sheet and disordered conformations of α gliadin are practically not affected by presence of gum arabic (described above). Therefore, the α helices are the main structures affected in the α gliadin−gum interactions at pH 3.0. These interactions could be partially hindered by excessive rigidity of the helical domains at higher pH of 4.0. On the other hand, they are completely hindered by protein denaturation at lower pH of 2.5. Indeed, we have previously reported[1] that, at pH 2.5, the α gliadin−gum arabic coacervation is almost no longer observed: At this pH value, interactions are no longer sufficient to allow the phase separation.

Possible Mechanisms of Protein−Polysaccharide Complex Coacervation. Concerning electrostatic attraction between positive charges of protein and negative charges of polysaccharide, the initial hypothesis was that it should be more important for pea globulin than for poorly charged α gliadin. This interaction is expected at pH 4.0, but appears less probable at the extremely acidic pH below 3.0, when the majority of carboxyl functions of polysaccharide are protonated ($pK_a \approx 4.7$) and rather favorable to hydrogen bonds.

For globulin−polysaccharide interactions, the increase of the band at 880 cm^{-1} (described above) is the strongest at pH 4.0 (Figure 5), thus indicating the importance of ionic interactions for this protein. In contrast, α gliadin−polysaccharide interactions are less dependent on an ionic mechanism, since the band at 880 cm^{-1} is stronger at pH 2.5. On the other hand, for both proteins studied here, conformational perturbations by gum arabic (Figure 4) are very strong at low pH values of 3.0 (α gliadin) and 2.75 (globulin). These results indicate that non-Coulombic interactions such as intermolecular hydrogen bonds are also important for stabilizing their respective complexes with the polysaccharide. Nevertheless, overall protein structure and charge profiles are responsible for the fact that the interactions involve different domains: mainly *β*-sheet in globulin and mainly α-helix in α gliadin.

Because of different geometry of water−carbonyl group interactions, the water hydration can be weaker to *β*-sheet than to α-helix structure, which may play a role in the network of the aggregates.[25] This suggestion is in agreement with our recent results on relaxation time measurements in NMR:[36] The mobility of water molecules in the environment of α gliadin on one hand and pea globulin on the other hand was quite different and crucially dependent on pH.

The results described in this paper are complementary to those of our previous study of interfacial rheological behavior of coacervate films at similar pH conditions.[35] It was concluded that the interfacial quality of the film was an important factor governing its aptitude to form a continuous and smooth layer around oil droplets. In summary, for pea globulin at pH 2.75, the coacervate layer showed a viscoelastic behavior, with an apparent elasticity lower than at pH 3.5. This might contribute to the better encapsulation rates observed for globulin at lower pH. The system α gliadin−gum arabic showed a higher viscoelasticity value at pH 3 than at pH 3.5, which also corresponded to the best encapsulation conditions. Hence, the rheological properties of the coacervate film appeared related to a certain extent to the interfacial rheological properties of the protein used in the coacervation process.

IV. Conclusions

Protein unfolding at pH values below the isoelectric point is observed through the decrease of both α-helix and *β*-sheet

Raman contributions, namely, in the amide I region. Pea globulin exhibits a particularly strong increase of disordered structure, whereas α gliadin keeps predominating α-helical signal.

When protein is coacervated with gum arabic, the conformations implied are mainly β-sheets in pea globulin and α-helix in α gliadin. Raman data indicate that the strongest protein−gum arabic interactions are observed at pH 2.75 for globulin and pH 3.0 for gliadin. At these pHs, the effect of gum arabic consists of stabilization of α-helical regions of both proteins. For α-helical regions of globulin, this effect of polysaccharide is weaker but accompanied with a decrease of random fraction down to the level close to that in the powder sample. In contrast, the β-sheet domains are not protected by gum arabic; moreover, in the case of globulin, these domains are even more perturbed by the protein−gum interaction than by pH.

The pH values discussed here are consistent with our previous results on the pH conditions corresponding to optimal complex coacervation.[1] Therefore, optimal coacervates are observed when (i) the pH-induced conformational perturbations of free protein structure are the strongest and (ii) when the compensation of these perturbations by gum arabic is the most pronounced.

From the analytical point of view, the present results demonstrate Raman microspectroscopy to be a powerful tool for studying the secondary structure perturbations in vegetal proteins upon complex coacervation.

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