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COATING LAYERS OF MAJOR STORAGE PROTEIN FROM ALEURONE CELLS OF BARLEY STUDIED BY ATOMIC FORCE MICROSCOPY

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Coating layers of storage protein from aleurone cells of barley (*Hordeum vulgare L.*) were studied by protein adsorption from different aqueous saline solutions on the solid support, e.g. glass or mica, and by tapping mode atomic force microscopy (AFM). The AFM images led to topographies that reveal nanometer details of surface structure of protein coating layers. The surface coverage of solid support and the structure of adsorbed protein layers were studied as function of adsorption time, protein concentration, pH and ionic strength. The structure of the adsorbed protein coating layers on glass is apparently ordered and some indications of long range order can be suggested presumably due to the electrostatic effects and to the surface and protein attraction.

INTRODUCTION

Seed proteins are classified into three groups, namely, storage proteins, structural and metabolic proteins, and protective proteins. The storage proteins of cereal grains have important nutritional importance for humans and they influence the utilization of the grains in food processing. Because of the economic and nutritional importance, the cereal storage proteins have been a major topic of research for many years with the aim of understanding their structures, control of synthesis, intracellular deposition within protein storage vacuoles or storage compartments, and the role in grain utilization.[1-5] Up to now, much less attention has been focused on physical and chemical characterization of adsorbed layers of seed storage proteins.

The changes in the structure of the adsorbed layers of major storage protein of aleurone cells of barley during the protein adsorption from its bulk aqueous saline dispersions on solid substrate is the main topic of the present paper. Such studies might bring light on the molecular structuring processes which might occur in vacuoles that store proteins.

The major storage protein from aleurone cells (noted PAC protein) of barley (*Hordeum vulgare L.*) was extracted and purified from the Himalaya cultivar of barley.[1] The major storage PAC protein component is soluble in dilute salt solutions and belongs to seed proteins classified as globulins based on their solubility in solvents.[4] The PAC protein has a common N-terminal amino acid sequence which was similar to that in the N-terminus of pea and bean vicilins, as well as cottonseed 7S globulins.[1]

The physical and chemical characterization of protein adsorption is the main step crucial for understanding the electrostatic effects among protein particles and their ordering in structured coating layers. Generally, protein adsorption can be studied by a wide range of instrumental methods.[6] However, calibration and other sources of uncertainty introduce potential errors into the characterization of adsorbed protein layers at different surfaces.

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In order to study the protein adsorption and molecular structuring processes in coating layers on solid surfaces the scanning probe microscopy (SPM) methods provide such capability. The first from such methods is scanning tunneling microscopy (STM) that allows a high resolution but it is limited to conducting solid substrates. The second one is the atomic force microscopy (AFM) which overcomes the principal problem of STM mentioned above. Also, AFM is an efficient tool to detect surface morphology and adhesion properties through tapping mode with an excellent spatial resolution.

The aim of this work is to study the coating layers of self assemblies of the PAC protein adsorbed on the solid surface. Our main focus is on the surface structure of dried protein layers and on the appearance of protein nanoparticles within the protein coating layers. To the best of our knowledge, this is the first report providing the adsorption of the PAC protein at a solid surface and includes nanoscale details on the structure of PAC protein layers.

In the following, we present the AFM study of the adsorption of the PAC protein on glass substrate as function of adsorption time and characteristics of aqueous solutions. The obtained results are showing the structure of the coating PAC protein layers indicating whether ordered arrays of such protein might be prepared by simple adsorption. The resulting data can also bring light on the protein adsorption process, as well as on the behavior of adsorbed protein coating layers.

EXPERIMENTAL SECTION

Materials and methods

The major storage protein component from aleurone cells of barley (PAC protein) was extracted and purified as previously described.[1] The PAC protein is a mixture of four closely related peptides of about 20-, 25-, 40- and 50 – kDa. These subunits are not associated by inter-chain disulphide bonds.[1] Furthermore, it was evidenced that strong hydrophobic interactions between the hydrophobic parts of these peptides contribute to the high stability of the PAC protein.[1]

The purified PAC protein powder was dissolved in ultra pure water of pH 5.6, in phosphate buffers of pH 7.2 and 0.15 NaCl, or in NaCl (0.5 M) aqueous solutions of pH 5.3, at different protein concentrations in the range from 0.0002 to 20 mg/liter of solution. The aqueous subphases in the absence and the presence of protein were prepared with ultra pure water of 18 Mohm cm^{-1} obtained by using an Elgastat system. The solid substrate for PAC protein adsorption was the optically polished glass.

The PAC protein coating layers on the solid substrate were prepared by immersing the substrate vertically in protein aqueous solutions at room temperature. After a chosen time, each substrate was withdrawn carefully from the protein solution, ensuring that the excess of protein molecules attached to the surface was washed out by rinsing the sample with the same aqueous solution but without protein or with highly purified water. Afterwards, the PAC protein sample was placed under a beaker to be dried slowly at room temperature and then, it was placed to be imaged by AFM – JEOL 4210.

The AFM imaging was usually performed in taping mode.[7-11] However, occasionally the contact mode AFM imaging was performed and the applied forces of less than 10^{-10} N were used. The 2D arrays were evidenced in both AFM operating modes and this indicates a high stability afforded by the ordered arrays within coating protein layers (Fig. 1). Also, a wide range of complementary methods including interfacial tension and surface pressure measurements and transmission electron microscopy (TEM)[11] on the PAC protein films have been used to support the structure of PAC protein layers. The disruption of the protein layer by the AFM tip in contact mode was insignificant and the images practically coincide with AFM tapping mode ones.

Thus, the given AFM images are performed by using AFM tapping mode on JEOL 4210 equipment. The AFM probes were triangular cantilevers with ultra-sharpened silicon nitride tips. The tips were on a cantilever with a resonant frequency in the range of 200 - 300 kHz and with a spring constant of 17.5 N/m. We used low scan rates of 1 Hz and high scan rates in the range of 20-30 Hz to detect noise artifacts. The scan angle was also changed in different directions to observe real images from those corresponding to noise. The AFM image consists of multiple scans displaced laterally from each other in y direction with 512 x 512 pixels. Low pass filtering was performed to remove the statistical noise without to loose the features of the sample. All AFM experiments were carried out under ambient laboratory conditions (about 20 ^{o}C) as previously reported.[7, 8]

RESULTS AND DISCUSSION

The major PAC protein is a holoprotein formed of four closely related subunits of molecular weight of about 20, 25, 40 and 50 kDa[1] and the subunits are strongly associated by hydrophobic interactions. The PAC protein adsorption at the air/water and oil/water interfaces has been studied (unpublished results) and indicates that PAC protein is a quite surface active biopolymer.

In the present contribution, several experimental variables have been found to affect both the structure of adsorbed protein layers on glass and the surface coverage with coating protein layers, namely, the pH, ionic strength and protein concentrations in aqueous solutions. Thus, the coating layers are obtained by direct

adsorption of protein particles on glass, immersed into the chosen aqueous phase of different protein concentrations.

The AFM observations are repeated on different areas from 10 x 10 μm^2 to 1 x 1 μm^2 of the same sample. The AFM images were obtained from at least five macroscopically separated areas on each sample. All images were processed using the standard procedures for AFM.[7-11]

The AFM tapping mode observation allows simultaneous acquisition of both topographic data (topographic image, Fig. 1, part 1) and material properties data (phase image, Fig. 1, part 2). Usually, the topographic images show the morphology of adsorbed protein coating layers. The phase images indicate modifications in surface properties of protein coating layers on solid substrate, such as elasticity, friction and adhesion.

The images in Figs. 1 demonstrate that, generally, the topographic (Fig. 1.1) and phase (Fig. 1.2) images appear to be complementary to one another showing the structural features of coating protein layers. The appearance of protein nanoparticles is well illustrated within the adsorbed protein layers (Fig. 1.1). The size of protein nanoparticles can be measured directly from AFM topographic (Fig. 1.1) image and the thickness (vertical) variations are estimated from cross section profiles through the topographic image as shown previously.[7-11]

A typical AFM image of the adsorbed coating layer of PAC protein on glass is shown in Fig. 1.1. The adsorption of PAC protein takes place from aqueous solutions of 20 mg of protein in a liter of highly purified water, containing 0.5 M NaCl, of pH 5.3. Sometimes, the protein nanoparticles are aggregated into clusters with protein molecules closely packed. Their heights are ranging from about 3.5 nm to 12 nm, clearly distinguishable from the glass surface roughness (less than 1 nm).

The apparent width of these protein nanoparticles is between 70 and 80 nm and in good agreement with the observed diameter of about 70 nm found from TEM micrographs for protein self-assemblies formed in aqueous solutions.[11] For TEM investigations the protein adsorption takes place from aqueous solutions directly on TEM grids covered by a collodion homogeneous thin film.

The AFM image, as given in Fig. 1.1, is taken after a period of two hours adsorption, which is long enough for glass surface saturation with protein nanoparticles. This image shows that PAC protein molecules are adsorbed as nanoparticles or clusters of nanoparticles closely packed on glass surface. Several structures are observed as bright spots and could be the larger aggregates of protein nanoparticles or protein colloidal particles. Also, small black regions probably uncoated with adsorbed protein layers are seen. A possible explanation of these regions might be the absence of adsorption due to some non-homogeneities of the glass surface.

The individual protein molecules are not easy to be identified within the protein nanoparticles or in protein colloidal particles. As shown above, the apparent widths of isolated nanoparticles are slightly larger than those measured by TEM. This is due primarily to the tip-broadening effect that occurs with tip of finite sharpness. In other words, it is a little compression of the adsorbed protein nanoparticles due to the applied force of the tip or due to the nanoparticles adhesion to the solid substrate.

Further, by taking the Fig. 1.1 as a reference image, the effects of several experimental variables on the characteristics of the coating protein layers are discussed, namely the influence of adsorption time (Figs. 2), protein concentrations (Figs. 3) and the ionic strength and pH values (Figs. 4).

Thus, from Figs. 2, it is to be noted that the structure of the adsorbed protein layer on glass is rather ordered and the glass surface saturation is reached at 2 hours adsorption time (Fig. 2.3) for protein adsorption from aqueous solutions of protein of 0.02 mg/liter of saline aqueous solution.

Analyzing the data given in Figs 3 and 4, it is to observe that the behavior of protein is complex generating larger nanoparticles with increasing bulk protein concentrations as shown in Figs. 4. Again, at 2 hours adsorption time, the protein nanoparticles appear to be adsorbed at the glass surface saturation.

Also, a qualitative study of these images shows that the AFM image, given in Fig. 4.1, is identical with the chosen reference image from Fig. 1.1. In addition, this AFM image shows that the protein nanoparticles are somehow well ordered within the adsorbed coating layers. This observation is plausible because the glass substrate is negatively surface charged and the PAC protein is positively charged at all pH's used and a strong adhesion of protein layers on the glass surface is achieved. Even more, the 0.5 M NaCl aqueous solutions, of pH 5.3, appear to be a favorable aqueous environment to generate stable well ordered nanostructured PAC protein layers adsorbed on the glass surface.

As seen in Figs. 4, the shape of protein nanoparticles within the coating layers (Fig. 4.1) is distorted particularly for the protein adsorption from highly purified water (Fig. 4.3) in the absence of electrolyte. Therefore, it is clear that the ionic strength of aqueous solutions can affect the adsorption of charged protein nanoparticles or protein colloidal particles by screening the electrostatic interactions, influencing the repulsion among particles, and increasing the solid substrate and protein attraction. Similar electrostatic effects were also evidenced in the case of ferritin adsorption from aqueous solutions on hydrophobic glass.[6]

Coming back to Figs. 1, the structure of the adsorbed PAC protein layer on glass can be characterized as uniformly distributed protein nanoparticles without an apparent spatial order or periodicity. However, the local ordering of adsorbed PAC protein nanoparticles can be analyzed by fast Fourier transformation, noted FFT, of the topographic AFM image, given in Fig. 1.1. The typical FFT image is given in Fig. 1.3 for the highly covered glass surface with adsorbed protein layers, as shown in Fig. 1.1. In FFT image (Fig. 1.3), there are some clearly defined peaks, which could indicate a spatial periodicity and an orientation ordering in the two-dimensional array as it was also found in the case of ferritin adsorption on glass.[6] This 2D arrangement was not seen under any other solution conditions. Also, this 2D array was not seen for the case of PAC protein adsorption on mica, which is a strong negatively charged surface.[11]

Thus, the ordering of PAC protein nanoparticles, within coating layers on glass, by adsorption of PAC protein from aqueous solutions can be realized with the condition that the surface charge is small or electrostatic effects are highly screened.

Generally, the utilization of self-assembly systems, wherein biomolecules spontaneously associate into reproducible nanoparticles or aggregates and supramolecular structures, represents one of the key challenges in nanobiotechnology. The potential application of coating layers in nanobiotechnology is based on the specific features of the 2D arrays composed of protein particles. The important physicochemical properties of coating layers are determined by functional groups on the protein coating layers which are arranged in well-defined positions. Many applications of protein adsorbed layers may depend on the capability of protein to crystallize into arrays in aqueous suspensions or on suitable surfaces (*e.g.* polymers, metals, silicon wafers) or at fluid interfaces (*e.g.* lipid films or liposomes).

In the future, the chemical structure of the coating protein layers adsorbed on different surfaces will be studied by FTIR and FTIR – attenuated total reflection (FTIR-ATR), Raman and SERS, and by surface plasmon resonance.[12] The structural characterization of proteins in two-dimensional (2D) crystals and their use to initiate 3D protein crystallization will be assessed by X-ray diffractions and advanced spectroscopy. Undoubtedly, the obtaining of ordered and stable protein adsorbed layers might have potential applications as diagnostic tools and biosensors.

CONCLUSIONS

Our studies showed that the PAC protein layers, at the solid surface saturation, are stable and they have a compact structure. These findings have led us to widely use of PAC protein in AFM studies of protein adsorption from aqueous solutions and its two-dimensional ordering and structuring at interfaces.

The PAC protein nanoparticles appear in the AFM images to be uniformly distributed within the coating layers on the glass substrate at all investigated pH values after sufficiently long periods (*e.g.* 2 hours) allowed for adsorption. The PAC protein shows a unique structural pattern in its adsorbed layers on the glass surface as visualized by AFM. Such molecular structuring processes might appear within vacuoles that store proteins during the grain development.

On the other hand, the structural basis found within PAC protein layers can generate complex supramolecular structures involving various classes of biological molecules, e.g., lipids, natural pigments or combination of those. Therefore, the globulin storage PAC protein might fulfill the key requirement as building blocks for the production of new supramolecular materials as required in molecular nanotechnology and biomimetics.

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