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# Self-Assembling Systems Based on Amphiphilic Poly-N-vinylpyrrolidone and Their Interaction with Model Proteins

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**Abstract**—Polymeric particles formed by stearoyl-poly-N-vinylpyrrolidone (PVP-stear) of $M_n$ = 2600 were obtained in aqueous solution, and their shape and size distribution were characterized. The size of the particles was shown to decrease with an increase in the ionic strength of the solution. Interaction of PVP-stear and its aggregates with model proteins (Bowman–Birk soybean proteinase inhibitor (BBI) and its hydrophobized derivatives) was studied. The possibility of inclusion of both native BBI and oleoylic derivative of BBI in the PVP-stear polymeric aggregates was investigated. It was established that polymeric particles with a diameter of 30 nm formed under certain concentration ratios between PVP-stear and poorly soluble dioleoyl BBI are capable of solubilization of dioleoyl BBI as well as prevention of its inactivation at low pH values.

*Key words*: self-assembling systems, colloidal particles, polymeric aggregates, stearoyl-poly-N-vinylpyrrolidone, Bowman–Birk soybean proteinase inhibitor

Various polymers are used in the development of protein drug delivery systems, as well as in increasing protein stability, providing prolonged and targeted action, which enhances the therapeutic efficacy of drugs [1-4]. The most promising for such systems are biocompatible and biodegradable polymers, which have been employed in injection and implant systems [5, 6].

There are different types of disperse polymer systems for drug delivery: microcapsules, vesicles, micelles [4, 7].

Micellar delivery systems based on amphiphilic water-soluble polymers provide growing interest because they allow solubilizing drugs and thereby increase their bioavailability. Depending on size of the particles, it is possible to regulate drug accumulation in different organs and tissues of the organism. Another advantage of using micelles is an ability of simple, quick, and reproducible production in large quantities [4, 8, 9].

Amphiphilic derivative of poly-N-vinylpyrrolidone (PVP-stear) with molecular weight of 2600 daltons, containing a terminal hydrophobic group (a residue of a long-chain stearic acid), was synthesized by radical polymerization of N-vinylpyrrolidone in the presence of functional mercaptans, followed by interaction with an acid [10]. The aim of this study was to investigate PVP-stear self-assembly processes in aqueous solutions and the interaction of model proteins with disperse systems based on the polymer.

Bowman–Birk soybean proteinase inhibitor (BBI) and its hydrophobized derivatives were used as model proteins. BBI is able to simultaneously inhibit trypsin and chymotrypsin [11] and also human leucocyte elastase [12]. BBI actively suppresses cell transformation *in vitro* and carcinogenesis *in vivo* [13]. However, the high therapeutic potential of this inhibitor is limited by its rapid clearance from the organism [14]. As we have demonstrated earlier, BBI formulations modified by unsaturated fatty acids derivatives (such as oleic, linoleic, α-linoleic acids) have better membranotrophic properties compared to the native protein [15], and also higher affinity to chymotrypsin and leucocytes elastase [16]. However, the solubility of BBI hydrophobized derivatives in aquatic medium is limited.

Thus, the investigation of possibility to increase the solubility of acylated BBI formulations by including them into colloidal systems formed by amphiphilic PVP-stear opens new approaches to the development of highly efficient systems for hydrophobized BBI delivery.

## MATERIALS AND METHODS

**Materials.** Trypsin and chymotrypsin (Merck, Germany), N-benzoyl-L-arginine ethyl ester hydrochloride, N-benzoyl-L-tyrosine ethyl ester, and Bowman–Birk soybean proteinase inhibitor (Sigma, USA), and

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Superose 12 (Pharmacia, Sweden) were used in this study.

Modified BBI, containing either one ($(ole)_1$BBI) or two ($(ole)_2$BBI) oleoyl residues were obtained as we described previously [17]. PVP-stear with a molecular weight of 2600 daltons was also synthesized according to a known technique [10].

The content of active sites in the trypsin preparation determined using *p*-nitrophenyl ether of *p'*-guanidinobenzoic acid titration [18] was 64% active by weight. The content of active sites in the chymotrypsin preparation determined using N-*trans*-cinnamoylimidazole titration [19] was 57% active by weight.

The self-assembly processes of PVP-stear in the absence and in the presence of BBI was studied by adding the corresponding amount of protein to PVP-stear solutions of various concentrations, both in purified water and physiological solution (0.15 M NaCl, pH 7.4). The solution was agitated using a KS 500 shaker (Labortechnik, Germany) at 100 rpm for 20 min; samples were taken and analyzed. To extract the protein from the colloidal system, the precipitation with threefold acetone volume was used (−20°C). The precipitate was centrifuged and dried. Purity of the protein was monitored electrophoretically. Electrophoresis of the protein and BBI mixtures with PVP-stear was performed in 15% polyacrylamide gels according to Reisfeld at pH 4.5 [20]. Protein content per lane was 6.5 μg.

Aggregation concentration ($C_{aggr}$) of PVP-stear was determined by the maximum bubble pressure technique with an MMN-240 micromanometer. Ethanolic solution with a density of $0.8095 \pm 0.005$ g/cm$^3$ was used as the manometer liquid. Reference liquid was poured into a double wall vial and thermostatted at 22°C. Distilled water or physiological solution (0.15 M NaCl, pH 7.4) was employed as the reference liquid. Aggregation concentration was determined as corresponding to the surface tension jump with an increase in polymer concentration.

Particle size was determined using the dynamic light scattering technique [21]. Monochrome irradiation from a 2 mW helium-neon laser (wavelength 632.8 nm) was passed through a thermostatted cuvette. Scattered at 90° angle light hit a photodetector consisting of a photomultiplier and a UNICOR-SP multichannel correlator (Sweden). Data processing was performed using the UNICOR-SP software.

Transmission electron microscopy was performed using a JEOL JEM-1000 equipment (Germany) at 20 kV voltage. For sample preparation, the droplet of solution was placed on polyvinyl formal carrier (0.2%, Sigma) applied on a copper grid.

Antitryptic activity of BBI formulations was determined according to relative esterase activity of trypsin remaining after incubation with an inhibitor. Trypsin solution (0.1 ml, 0.1 mg/ml in 0.001 M hydrochloric acid) was placed into the cuvette. Then, 0.1 to 0.7 ml of inhibitor with amphiphilic PVP-stear at the corresponding dilution was added, and the total volume of the mixture was adjusted to 0.8 ml by adding 0.05 M Tris-HCl, pH 8.0. The mixture was incubated for 10 min at 25°C. Thereafter, 0.2 ml of 1.5 mM BAEE solution in Tris-HCl, pH 8.0, was added to the cuvette and the change in optical density was registered for 1-5 min at 253 nm using a Shimadzu UV-265 FW spectrophotometer (Japan).

Gel permeation chromatography of BBI formulations was performed with an FPLC unit (Pharmacia) using a Superose 12 HR 10/3 column (1.5 × 35 cm). Tris-HCl buffer (0.05 M, pH 8.5) with 0.15 M NaCl was employed as an eluent. The sample (200 μl) was loaded on the column, and then eluted at 0.6 ml/min flow rate. Protein content in the fractions was monitored by the absorbance at 280 nm.

## RESULTS AND DISCUSSION

This study investigated the possibility of self-assembling of amphiphilic PVP-stear in aqueous solutions. Dependence of surface tension value δ on amphiphilic polymer concentration (Fig. 1) was used to determine the aggregation concentration. Values of aggregation concentration ($C_{aggr}$) in water and in physiological solution were 0.41 and 0.25 mg/ml, respectively. It is well known that hydrophobic interactions intensify with an increase in ionic strength of a solution, and therefore the decrease in $C_{aggr}$ value in physiological solution can be attributed to the increased interaction between terminal stearoyl groups in polymer chains. Particle size of the obtained aggregates at PVP-stear concentration ≥0.5 mg/ml was studied by the dynamic light scattering

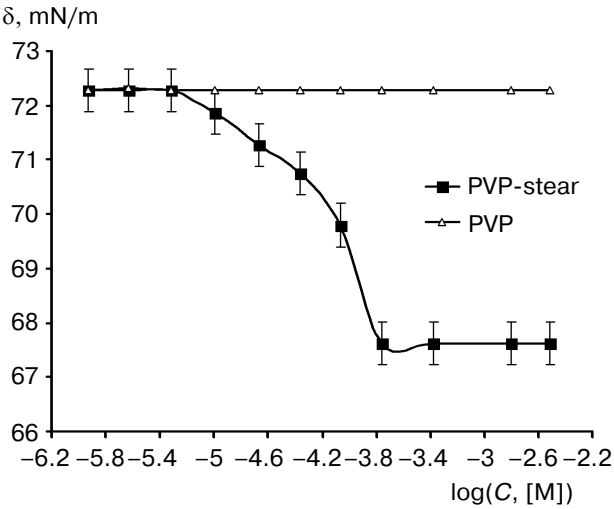


**Fig. 1.** Isotherm of surface tension for aqueous solutions of amphiphilic PVP-stear.

**Table 1.** Particle sizes for PVP-stear aggregates determined using the dynamic light scattering technique

| [PVP-stear], mg/ml | Average particle size, nm | |
|---|---|---|
| | water | physiological solution |
| < 0.05 | no particles | no particles |
| ~0.5 | 540 ± 20 | 300 ± 10 |
| > 0.5 | 560 ± 20 | 300 ± 10 |

technique (Table 1). It was illustrated that particle formation in water and physiological solution does not occur at amphiphilic PVP concentration, which is 10 times lower than $C_{aggr}$. The average diameter of the particles is constant and equal to ~550 nm in water and to ~300 nm in the physiological solution at polymer concentration ≥ $C_{aggr}$. Such a decrease in particle size can be explained by increased hydrophobic interactions in the solution with higher ionic strength, which leads to the formation of more compact polymeric structure. It should be noted that diameters of those particles are presented in Table 1, which contribute most to the size distribution of the particles, i.e., there are both small 80 nm particles and large 440 nm particles present in PVP-stear physiological solution (Fig. 2). Assuming a linear structure of the polymer, the maximal size of its macromolecule in the first approximation will be 10 nm, and micelle particle diameter should not exceed 20 nm. Correlation of this value with the data obtained using dynamic light scattering shows that the system contains particles larger than micelle sized. Therefore, in this case the term "aggregation concentration" is used instead of "critical micelle concentration".

Particles formed in the system were investigated using transmission electron microscopy (TEM, Fig. 3). The TEM microphotographs reveal that the particles have spherical shape. The possibility of formation of both 100 nm particles and polymer aggregates of ~400 nm in physiological solution at PVP-stear concentration > $C_{aggr}$ was demonstrated. Hence, it was established that amphiphilic polymer based on PVP forms large macromolecular associates of spherical shape.

Electrophoresis of BBI mixture with PVP-stear reveals the appearance of a protein band with higher electrophoretic mobility than the native protein, which indicates the presence of PVP-stear bound BBI.

Tables 2 and 3 present the results of BBI and PVP-stear mixture investigated by dynamic light scattering. As seen from these data, increase in native BBI concentration from 0.5 to 2.0 mg/ml results in decrease in particle size from 450 to 300 nm. The presence of protein probably compacts and arranges the structure of colloidal particles. The TEM data demonstrated that the particles formed are of spherical shape. It should be noted that contrary to the native BBI, $(ole)_2$BBI initiates the formation of mixed aggregates at low PVP-stear concentration in aqueous solution. Such difference in interaction of two BBI preparations with the polymer can be associated with an increase in protein affinity toward amphiphilic PVP-stear due to increase in the hydrophobic interactions between them caused by incorporation of two oleoyl residues into the BBI molecule. It should be emphasized

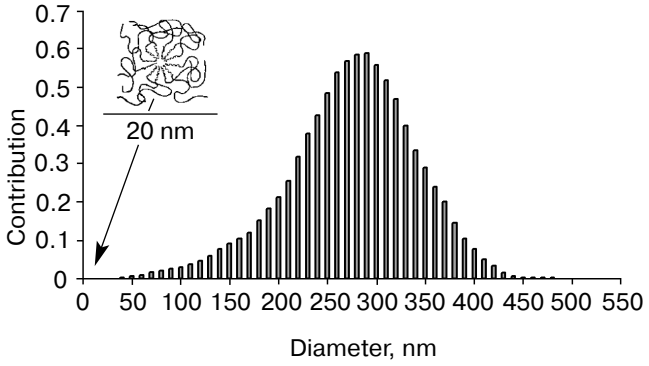


**Fig. 2.** Size distribution of particles in physiological solution for PVP-stear (5.0 mg/ml).

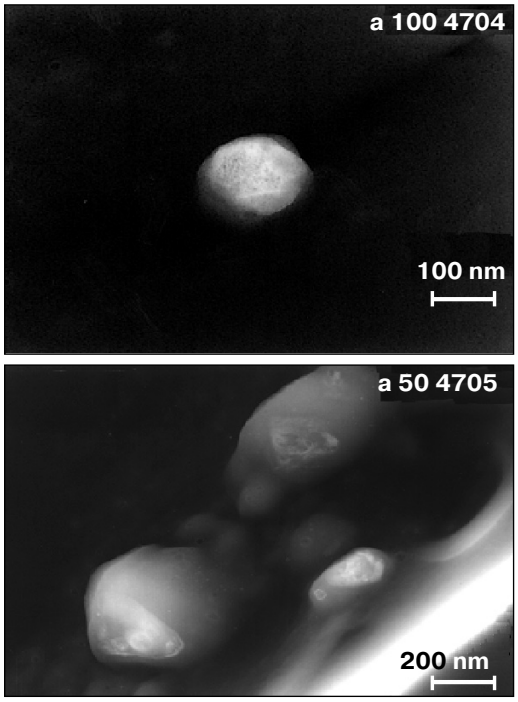


**Fig. 3.** Microphotographs of particles obtained using the TEM technique, [PVP-stear] = 0.5 mg/ml in physiological solution.

that at certain ratio of PVP-stear and $(ole)_2$BBI concentrations in the mixture, the protein becomes completely soluble in an aqueous medium. Table 2 shows that at constant PVP-stear concentration (0.5 mg/ml) the increase of $(ole)_2$BBI concentration from 0.5 to 10 mg/ml results in the decrease in the average particle size from 370 to 30 nm. In addition, particle size of 30 nm is comparable with an expected micelle size for the polymer.

It can be assumed that in this case the destruction of large polymer assembles takes place along with the formation of colloidal particles, micelles, solubilizing the hydrophobized protein. Interaction forces between the polymer chains dominate at low $(ole)_2$BBI concentration in the polymer mixture; however, with an increase of hydrophobized protein concentration the main contribution comes from the hydrophobic interactions between protein fatty acid residues and the polymer, which leads to the formation of micelles instead of mixed polymer aggregates. This suggestion was proved by gel permeation chromatography data (Fig. 4). As seen in Fig. 4, mixture II obtained under inclusion of a small amount of hydrophobized protein into the polymer aggregates (0.5 mg/ml) contains fractions with elution volumes ($V_{el}$) 6.6 and 15.0 ml as well as a fraction corresponding to unbound protein ($V_{el}$ = 18.6 ml). With an increase of $(ole)_2$BBI concentration in mixture III (when the com-

**Table 2.** Size of mixed polymer aggregates of PVP-stear in water in the presence of BBI formulations

| PVP-stear, mg/ml | BBI, mg/ml | Average diameter, nm | $(ole)_2$BBI, mg/ml | Average diameter, nm |
|---|---|---|---|---|
| 0.05 | 0 | no particles | 0 | no particles |
| | 1.0 | no particles | 1.0 | 480 ± 20 |
| | 2.0 | no particles | 2.0 | 460 ± 20 |
| 0.5 | 0.5 | 430 ± 20 | 0.5 | 370 ± 20 |
| | 1.0 | 370 ± 20 | 1.0 | 30 ± 10 |
| | 2.0 | 300 ± 20 | 2.0 | 30 ± 10 |
| | | | 10.0 | 30 ± 10 |
| 5.0 | 0.5 | 450 ± 20 | 0.5 | 430 ± 20 |
| | 1.0 | 430 ± 20 | 1.0 | 360 ± 20 |
| | 2.0 | 370 ± 20 | 2.0 | 270 ± 20 |
| | | | 10.0 | 30 ± 10 |

**Table 3.** Size of mixed polymer aggregates of PVP-stear in buffer 0.05 M Tris-HCl, 0.15 M NaCl, pH 8.5, in presence of hydrophobized BBI derivatives

| PVP-stear, mg/ml | $(ole)_1$BBI, mg/ml | Average diameter, nm | $(ole)_2$BBI, mg/ml | Average diameter, nm |
|---|---|---|---|---|
| 0.05 | 0 | no particles | 0 | no particles |
| | 1.0 | no particles | 1.0 | 430 ± 20 |
| | 2.0 | no particles | 2.0 | 400 ± 20 |
| 0.5 | 0 | 310 ± 20 | 0 | 310 ± 20 |
| | 0.5 | 280 ± 20 | 0.5 | 240 ± 20 |
| | 1.5 | 220 ± 20 | 1.5 | 30 ± 10 |

plete dissolution of the hydrophobized protein occurs) the chromatogram contains only one peak ($V_{el}$ = 16.2 ml), different from that for $(ole)_2$BBI formulation, and the peaks corresponding to macromolecular fractions of polymer bound protein. Dynamic light scattering demonstrated that the size of colloidal particles in this fraction is ~30 nm (Table 3).

The BBI molecule hydrophobization effect on the inclusion into colloidal structures formed by PVP-stear can be estimated using the data shown in Table 3 and Fig. 5. Thus, the inclusion of monoacylated BBI formulation instead of $(ole)_2$BBI into amphiphilic polymer solution (0.05 mg/ml) does not result in the formation of polymer associates. The hydrophobicity of $(ole)_1$BBI is probably not sufficient for triggering the micelle formation by the amphiphilic PVP-stear. However, similarly to the case of $(ole)_2$BBI inclusion into the polymer aggregates, a tendency for a decrease in particle size with an increase in protein concentration is observed.

Figure 5 shows the data of gel permeation chromatography obtained for amphiphilic PVP-stear mixture with various concentrations of $(ole)_1$BBI. It can be seen that there are both large polymer assemblies ($V_{el}$ = 6.6 and 10.2 ml) and hydrophobized protein itself ($V_{el}$ = 19.1 ml) present in the mixture when $(ole)_1$BBI (0.5 mg/ml) is added to the polymer aggregates. With an increase in $(ole)_1$BBI concentration 3 times in the mixture, rearrangement of the aggregates into particles of smaller size ($V_{el}$ = 7.6 ml) is observed, which corresponds well to the dynamic light scattering data (Table 3). It should be mentioned that there is a peak present in the polymer mixtures with $(ole)_1$BBI, which (as shown for $(ole)_2$BBI) corresponds to the micelle particles ($V_{el}$ = 16.2 ml). Therefore, there are both large associates and small-sized colloidal particles (~30 nm) present in the mixture with the inclusion of monoacylated BBI derivative into the polymer aggregates. It is important to emphasize that unlike $(ole)_2$BBI, the complete dissolution of $(ole)_1$BBI in the investigated range of protein and amphiphilic polymer concentration was not observed.

It is known that during the solubilization process the substance is included into the particle nucleus, and its contact with a surrounding media is practically impossible. To obtain an indirect confirmation of the solubilization process, the denaturing influence of low pH on $(ole)_2$BBI in aquatic medium was investigated along with the influence on $(ole)_2$BBI included in the polymer aggregates of different size, and later extracted from this

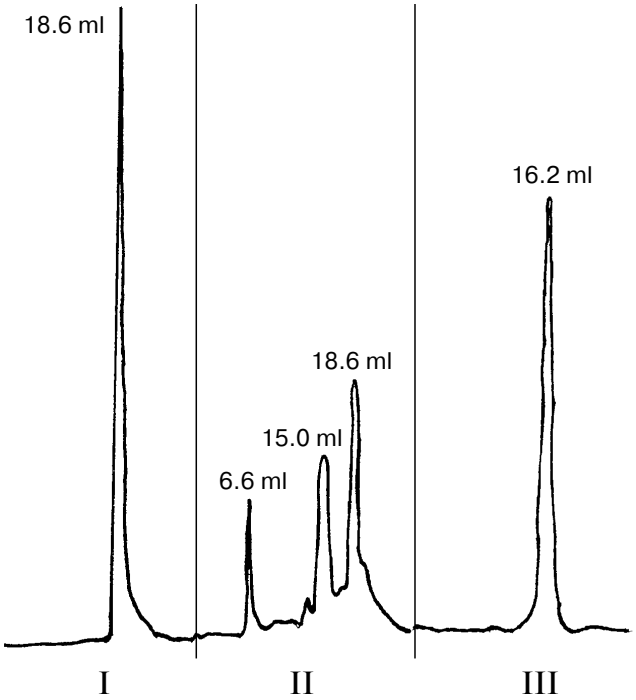


**Fig. 4.** Gel permeation chromatography on Superose 12 carrier (0.05 M Tris-HCl, 0.15 M NaCl, pH 8.5): I) $(ole)_2$BBI (0.5 mg/ml); II) $(ole)_2$BBI (0.5 mg/ml) + PVP-stear (0.5 mg/ml); III) $(ole)_2$BBI (1.5 mg/ml) + PVP-stear (0.5 mg/ml).

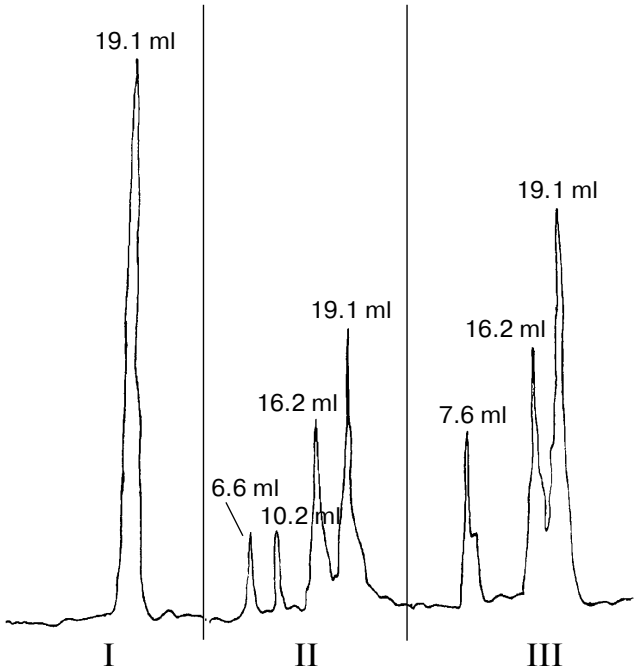


**Fig. 5.** Gel permeation chromatography on Superose 12 carrier (0.05 M Tris-HCl, 0.15 M NaCl, pH 8.5): I) $(ole)_1$BBI (0.5 mg/ml); II) $(ole)_1$BBI (0.5 mg/ml) + PVP-stear (0.5 mg/ml); III) $(ole)_1$BBI (1.5 mg/ml) + PVP-stear (0.5 mg/ml).

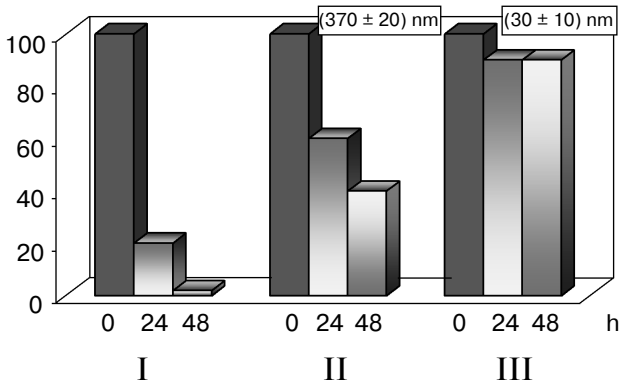


**Fig. 6.** Change in $(ole)_2$BBI antitryptic activity in the presence and in the absence of PVP-stear after incubation of the sample at pH 1.4: I) $(ole)_2$BBI (0.1 mg/ml); II) PVP-stear (0.5 mg/ml) + $(ole)_2$BBI (0.5 mg/ml); III) PVP-stear (0.5 mg/ml) + $(ole)_2$BBI (1.5 mg/ml).

mixture. Figure 6-I illustrates that $(ole)_2$BBI formulation loses 80% of its antitryptic activity during 24 h and is completely inactivated during two days. However, protein inclusion in the polymer aggregates facilitates the preservation of its activity (Fig. 6-II). Protein activity decreases by 60% during 48 h in the mixture of amphiphilic polymer with a protein, mainly consisting of large associates. It can be assumed that the protein fraction that is not included in the particle nucleus and therefore is able to contact the aqueous medium inactivates in this case. At the same time, the formation of colloidal particles (30 nm micelles) results not only in increased $(ole)_2$BBI solubility, but also in practically complete preservation of its inhibitory antiproteinase activity (80%) at pH 1.4 (Fig. 6-III). Obviously, this effect can be explained by the lack of interactions between hydrophobized protein and the solvent, which is additional proof of complete $(ole)_2$BBI solubility, correlating well with the data obtained by gel filtration of BBI, where the chromatogram (Fig. 4) for this composition of mixture III contains only one peak ($V_{el}$ = 16.2 ml). On the other hand, slight decrease (approximately 20%) of hydrophobized protein antitryptic activity can be caused both by the conformational changes of $(ole)_2$BBI occurring in the course of micelle formation with its subsequent embedding into the hydrophobic nucleus of the colloidal particle, and by the possibility of partial contact between the protein and the aqueous medium.

Therefore, polymer aggregate formulations of PVP-stear either containing or lacking the native and hydrophobized soybean proteinase inhibitor were obtained and characterized. The difference in affinity of the proteins with various numbers of oleoyl residues toward the amphiphilic polymer PVP, displayed in the initiation of particle formation as a result of the interaction between $(ole)_2$BBI and the polymer at the concentration below the aggregation concentration, was detected. It was illustrated that the formation of micelle-sized particles (30 nm) completely solubilizing the protein and preventing its inactivation is possible by the inclusion of hydrophobized proteins into the associates of amphiphilic PVP-stear.

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