Introduction Glycinin is a storage soybean protein belonging to the legumin family (11S globulin fraction). Legumins have high nutritional value, however, due to the peculiarities of molecular structure (high stiffness, low solubility near the isoelectric point) and, consequently, not enough high-functional properties, their use is limited. One of the most soft and promising ways to improve the functional properties of proteins is limited proteolysis. It is caused by the presence in a substrate molecule bonds (sites) are sensitive to attack with proteolytic enzymes. Limited proteolysis slows down in descending order with the formation of relatively stable high molecular weight product. The reorganization of protein structure by limited proteolysis leads to a change in its physico-chemical and functional properties. The purpose of this study was to establish the interrelationships between the change in molecular parameters of glycinin as a result of limited papain hydrolysis and a change of its thermostability, surface behaviour at the air/water interface (surface activity, the dynamics of formation and dylatation properties of adsorption layers). Materials and methods Samples of intact glycinin - 11S globulin fraction of soybean Glycine max (glycinin) and its modificate, obtained by limited proteolysis with papain (glycinin-P),were obtained according (1). The protein was stored in a saturated solution of ammonium sulfate at 5°C. Solution preparation. The samples of protein solutions were prepared by equilibrium dialysis against 0.05 M phosphate buffer pH 7.6 and 0.5 M NaCl. Solutions before the measurements were centrifuged at 20,000 g for 1 h and filtered through a cellulose membrane Millipore filters with a pore diameter of 0,22μm. The protein concentration in solutions was determined by microbiuret method (2). To prevent microbial contamination 0.02% NaN3 was added, to reduce the disulfide exchange - 0.1% mercaptoethanol. Laser light scattering. Determination of molecular parameters (molecular weight, second virial coefficient, hydrodynamic size, ξ-potential) was performed by static and dynamic laser light scattering using equipment Zeta Sizer Nano (ZEN 3600) (Malvern Instruments Ltd., UK), equipped with a 4 MW He-Ne laser (λ0 = 632,8 nm). The measurements were performed at 25°C and fixed scattering angle of 173°. A sample solution was filtered through a Millipore filter of 22 μm into a 1 cm measuring cuvette, which was preliminary with washed ~1mL of the filtrate. The time of temperature equilibration of the cuvette inside the instrument was 10 min. The final hydrodynamic radius distribution of the sample was found by averaging 10 measurements, each of which was a result of 10−16 scans. Small-angle X-ray scattering. Intensity of small-angle X-ray scattering of glycinin and glycinin-P solutions was measured at the ambient temperature ~25oC with the diffractometer described elsewhere [3] in the range of S values from 0.15 to 3 nm-1, where S=(4psinq)/l, q – half of a scattering angle, l – CuKa wavelength (0.1542 nm). The buffer used was 0.05M phosphate buffer saline (pH7.6) containing 0.5M NaCl, 0.02% NaN3 and 0.1% 2-merkaptoethanol. Protein concentration was ~30 mg/ml. Data processing including smoothing and desmearing was done as in [3]. The protein radius of gyration Rg was determined with the PRIMUS software [4] utilizing intensity I(S) approximation with the

Guinier formula [5]: I(S) = I(0)exp{-(SRg)2/3} (1) Differential scanning microcalorimetry. Determination of the thermodynamic parameters of thermodenaturation carried out by the method of adiabatic differential scanning microcalorimetry with using of microcalorimeter DASM-4 (BIOPRIBOR, Russia) within the temperature range 10−130 °C at a heating rate of 2 K min−1 and an excess pressure of 0.25 MPa. The primary data processing and conversion of the partial heat capacity of protein into the excess heat capacity function of the denaturation transition was performed using the standart Wscal software. The baseline in the transition area was obtained by a spline interpolation. The maximum temperature of the excess heat capacity curve was taken as the denaturation temperature, Td. The denaturation enthalpy, ΔHdcal, kJ/mol, was determined by integration of the excess heat capacity function. Dynamic tensiometry and dylatometry. Investigation of the surface activity, the dynamics of formation and dylatometric properties of adsorption layers of intact and modified proteins at the air/water interface were carried out on droplet tensiometer Tracker (TRACKER, IT Concept, Longessaine, France). Measurements were made in thermostated cuvette at 25 ± 0.1°C. Two-dimensional complex modulus of elasticity E of the adsorption layer is determined from the measured values of surface tension when the load is applied at the interface, which varies sinusoidally. Measurements of rheological properties produced after the formation of a sufficiently stable adsorption layer within 60-70 000 seconds. Fluctuations in the surface area of the bubble produced with an amplitude of 3%. The series of 3-5 consecutive loads of active and passive cycles at constant amplitude and frequency were carried out. The range of frequencies used was 0,001-0,02 Hz. Results and Discussion Glycinin and glycinin-P molecular parameters Table 1 shows the experimental values of the molecular structure parameters of the intact and modified glycinin (molecular weight, second virial coefficient, effective hydrodynamic radius, radius of gyration, sedimentation coefficient, frictional ratio, diffusion coefficient, respectively). Obviously, during the limited proteolysis some parameters of glycinin reduce: molecular weight, the diffusion coefficient, effective hydrodynamic size, and to a lesser extent - the radius of gyration. The magnitude of frictional relationship suggests lowering the degree of asymmetry of the modified protein molecules compared with intact. It was also established that glycinin-P molecule is characterized by a lower charge density (lower absolute value of ξ-potential: -17.2 mV comparatively to -20.3mV of glycinin) and a lower affinity to the solvent, namely, the value of B2 of glycinin-P is lower than the B2 of the intact protein molecule (Table 1). Table 1 - Molecular parameters of intact and modified by limited papain hydrolysis glycinin. Conditions: 0.05 M phosphate buffer, pH 7.6, 0.5 M NaCl; temperature of 25° C Sample Mw, kDa B2·10-4 mL•mol/g2 Rh, Nm Rg, nm S ·1013, c f/f0* f/f0** D·107, cm/sec Glycinin 362±21 3,57±0,21 5,69±0,18 4,22 11,9 1,35 1,43 4,06 Glycinin-P 269±24 1,64±0,18 5,16±0,15 4,15 11,8 1,24 1,26 4,38 f/f0* - calculated as the ratio of hydrodynamic radius to the radius of gyration; f/f0** - calculated on the basis of the

equation f/f0 = Smax / S = 0.00361M2/3/S [6]. Table 2 summarizes our obtained glycinin and glycinin-P thermodynamic parameters of denaturation (denaturation temperature, enthalpy, heat capacity increment, the maximum heat capacity, Gibbs free energy, the parameter of cooperativity, respectively). From the table it follows that glycinin-P thermodynamic parameters of denaturation are lower than that of the intact glycinin, indicating a destabilization of the molecules by limited proteolysis. Denaturation process is less cooperative (increased value of the parameter of cooperativity). Increase the heat capacity increment of glycinin-P compared with glycinin means the growth for the solvent accessible hydrophobic surface. This result correlates with a decrease in the thermodynamic affinity of the solvent glycinin molecules (lower B2) during proteolysis (table 1). Table 2 - Thermodynamic parameters of glycinin and glycinin-P denaturation. Conditions: 0.05 M phosphate buffer, pH 7.6, 0.5 M NaCl; scan rate 2 deg/min, pressure 0.2 MPa, protein concentrations - 2 mg/ml Sample Td, K ΔHdcal,·10-3 kJ/mol ΔCp, kJ/mol·K Amp, kJ/mol·K ΔGd,•102 kJ/mol ΔT= ΔHdcal/Amp Glycinin 370,1 12,6 151 1406 13,0 9,0 Glycinin-P 364,9 9,2 173 763 7,5 12,0 It is seen that observed changes in glycinin molecular characteristics as a reslt of limited proteolysis with papain are favorable for the increase of its surface activity and the rate of formation of the adsorption layers. Surface activity and the dynamics of formation of adsorption layers at the air/water interface Dynamic surface tension curves of adsorption layers formed by glycinin and glycinin-P at the air/water solution interface with different protein concentrations in phosphate buffer 0.05 M (pH 7,6) are shown in Figure 1. Shape of the curves is typical for globular proteins. Dependence for both proteins at low concentrations (0.001 and 0.01 mg/ml) are characterized by an S-shaped form, which can be divided into 3 main sections - an induction period (the initial part of the relatively slow decrease of surface tension), part of a significant reduction in surface tension (postinduction period) and the stationary phase, in which the tension reaches a minimum value and subsequently remains practically unchanged. It is known that the presence of the induction period (data are not shown) suggests that the adsorption process is controlled by the diffusion stage of the protein macromolecules from the bulk to the interface. When the concentration of proteins in solution is 0.001 mg/ml, the induction period is 1400 seconds for glycinin and 600 sec for glycinin-P (data is not presented). The decrease of the induction period in the case of glycinin-P is due to the higher rate of diffusion of the molecules caused by the lower molecular weight and a smaller effective hydrodynamic size. With the increase of the volume concentration of the protein value of the induction period is reduced [7]. In our case, at a constant bulk protein concentration of 0.1 mg/ml, the curves σ(τ) show only a post-induction and the final stages of adsorption. At the higher concentrations the "induction" period is absent, i.e. diffusion of macromolecules from the bulk solution to the interface is instantaneous. At the final stage of adsorption already formed adsorption layer begins to act as a repulsive barrier with respect to the macroion, which reaches the surface. This is

manifested in a gradual decrease in the rate of dσ/dτ. The decrease of surface tension after long periods of time indicates that the adsorbed macromolecule is controlled by diffusion of the active segments of its own redistribution in the adsorption layer. Increasing the rate of glycinin-P adsorption of dσ/dτ, as well as a lower value of the quasi-equilibrium values of surface tension of its solutions as compared to glycinin observed throughout the concentration range of proteins. The observed effects correlated with changes in molecular parameters: a lower charge density, higher surface hydrophobicity and lower conformational stability of glycinin-P comparatively to glycinin. We can assume that glycinin-P is able to generate more perfect adsorption layers. Effect of protein concentration on the expansion joint properties of adsorption layers at the air/solution interface The study of glycinin and glycinin-P rheological properties of adsorption layers was carried out by applying a small longitudinal perturbations ΔA (t) to the surface of an air bubble sinusoidally. ε (t) = εa • exp (iωt), (2) where ε = ΔA / A - relative longitudinal deformation of the layer, εa - the amplitude of the strain) at a constant frequency of ω. The deformation causes a change in surface tension. -Δσ (t) = σa • exp (iωt + φ)), (3) where φ - the phase angle (8). In the field of linear viscoelasticity complex modulus of elasticity can be expressed as follows: , (4) where E'(ω) and E"(ω) - real (modulus of storage) and imaginary (modulus of lost) parts of which are dependent on the applied frequency ω and define conservative and dissipative nature of the rheology of adsorption layers, respectively. Fig. 2 shows the frequency dependence of the applied compression - dilatation strain of the real E' and imaginary E" components of the quasi-equilibrium (formed during the 70 thousand sec.) complex viscoelastic modulus of glycinin and glycinin-P adsorption layers at various protein concentrations. In the whole frequency range both intact and modified forms of glycinin are characterized by a symbatic change in complex module and its real part (E'), and φ> 0 (data not shown), value of E' >> E", and E' is weakly dependent on ω. This type of rheological behavior is considered as solid-like. Fig. 1 - Dynamic surface tension curves of adsorbed layers of glycinin (dashed lines) and glycinin-P (solid lines) molecules. Protein concentrations: 0.001 mg / ml (black lines), 0.01 mg / ml (1, 1’), 0.1 mg / ml (2, 2’), 1.0 mg / ml (3,3’). Solvent - 0.05 M phosphate buffer pH 7,6, 0.5 M NaCl, 0,02% NaN3. Temperature: 25°C The differences in the parameters of the structure between glycinin and glycinin-P cause differences in the viscoelastic properties of adsorbed layers and the nature of their dependence on protein concentration. So over the entire frequency range and protein concentrations the observed higher values of E' of adsorption layers are higher in case of glycinin-P. Apparently, more compact and labile structure and a lower charge density of the glycinin-P molecules reduce the energy barrier and form a more perfect adsorption layer, providing a greater number of contacts between the macromolecules. More high surface hydrophobicity of glycinin-P molecules is also favorable for the stabilization of the adsorption layer due to hydrophobic interactions between macromolecules within the layer. Fig. 2 - The real and imaginary components of the dilatation modulus of

glycinin viscoelastic adsorption layers at different concentrations of proteins in solution. Module of conservation: (1) - 1.0 mg/ml, (2) - 0.1 mg/ml, (4) - 0.01 mg/ml, (3) - 0.001 mg/ml. Loss modulus: 1.0 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml. Conditions: 0.05 M phosphate buffer, pH 7,6, 0.5 M NaCl Fig. 3 - The real and imaginary components of the dilatation modulus of glycinin P viscoelastic adsorption layers at different concentrations of proteins in solution. Module of conservation: (1) - 1.0 mg/ml, (2) - 0.1 mg/ml, (3) - 0.01 mg/ml, (4) - 0.001 mg/ml; Loss modulus: (5) - 1.0 mg/ml, (6) - 0.1 mg/ml, (7) - 0.01 mg/ml, (8) - 0.001 mg/ml. Conditions: 0.05 M phosphate buffer, pH 7,6, 0.5 M NaCl Conclusion Thus, it was found that limited proteolysis with papain leads to a change in glycinin molecular parameters, namely, decrease of molecular mass, radius of gyration, hydrodynamic size and thermodynamic stability. At the same time the surface hydrophobicity of glycinin molecule increases. It is displayed in enhance of the heat capacity increment and in lowing of the second virial coefficient. As a result a significant growth of its surface activity is observed. Increase of the rate of formation and viscoelasticity of adsorption layers at the air/water interface are caused by decrease in diffusion coefficient, which in turn is caused by decreasing of molecular mass and hydrodynamic size. The interrelation between molecular parameter changes and glycinin surface behaviour is established. These changes are favorable for improving of the glycinin functional properties.