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ORIGINAL CONTRIBUTION

R. Borbás
S. Turza
J. Szamos
É. Kiss

Analysis of protein gels formed by interfacial partitioning

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R. Borbás · É. Kiss (✉)
L. Eötvös University
Department of Colloid Chemistry
P.O. Box 32, 1518 Budapest 112, Hungary
e-mail: kissevak@ludens.elte.hu
Tel.: +36-1-2090555; Fax: +36-1-2090602

S. Turza · J. Szamos
Central Food Research Institute
P.O. Box 393, 1537 Budapest, Hungary

Abstract A special combination of alcoholic precipitation and salting out of proteins was described as three-phase partitioning (TPP). Proteins are accumulated in a coherent middle phase in the partitioning system composed of *tert*-butanol, ammonium sulphate and water. Partitioning systems with various ratios of components were investigated. The composition and the mechanical properties of the middle phase were determined in model systems using bovine serum albumin or ovalbumin. Quantitative analysis revealed that beside the protein content (2–10wt%) other components of the middle phase formed two immiscible liquid phases. The stress–deformation relationship obtained by uniaxial compression showed the elastic behaviour of the middle phase. The results suggested that the middle phase formed in TPP is an emulsion gel. A possible mechanism of the formation of an emulsion gel was outlined.

Key words Three-phase partitioning · Emulsion gel · Protein separation · Stress–deformation behaviour of bovine serum albumin and ovalbumin gels

Introduction

Isolation of proteins is still a difficult task especially their separation from multicomponent systems. Various methods have been developed in the last decades but none of them could be applied generally. Several of these separation techniques are expensive and need pretreatment or precleaning of samples of natural origin.

Three-phase partitioning (TPP) was reported first in 1972 [1] as a method for separating enzymes from crude extracts. It proved to be a quick and easy way of enrichment of proteins from complex mixture of materials with various natures using only a few simple compounds, such as *tert*-butanol, ammonium sulphate and water. TPP can be described as a combination of the traditional salting out and alcoholic precipitation of proteins: it is based on systems consisting of two immiscible liquid phases made up of *tert*-butanol, ammonium sulphate and water. When the system contains protein, they form a third, so-called middle phase between the two immiscible phases. Water is miscible with *tert*-butanol in any proportion, but the mixture separates into two liquid phases in the presence of a certain amount of electrolyte. The upper phase contains mostly alcohol and some water with ammonium sulphate only in traces. The lower phase is an aqueous solution of electrolyte with a low *tert*-butanol content.

Applying phase partitioning to proteins led to the appearance of a gel-like disc (a third phase) between the two liquid phases [2]. Most of the proteins accumulated in the third or middle phase, the upper phase contained nonpolar compounds, while polar components remained dissolved in the lower aqueous phase. The practical advantage of that empirical method besides its simplicity was that accumulation of proteins could be achieved by their separation from components of other types in a one-step process. TPP was suggested as a preliminary purification process used for yielding or eliminating the great amount of protein from a crude multicomponent mixture.

TPP was successfully applied to prepare high-activity horseradish peroxidase [3]. The enzyme separation from extract of horseradish was performed by TPP, and then the enzyme accumulated in the middle phase was further purified by ion-exchange chromatography. TPP was also suggested as a possible tool in the characterisation of pork and beef drips [4].

In order to exploit the full potential of the TPP method it is necessary to understand the processes leading to the protein separation. Understanding the mechanism of TPP as well as the effect of various parameters on the yield could extend its application. The parameters affecting the behaviour of proteins in TPP were studied on model systems first by Pike and Dennison in 1989 [5]. The influences of the molecular mass and the hydrophobicity of proteins as well as that of the temperature and the pH on the partitioning process were investigated. It was shown that the amount of protein precipitated into the third phase depended on the initial protein concentration in a unique way. Furthermore, formation of a protein–*tert*-butanol complex was presumed because the protein-rich phase floated as a middle layer in contrast to the sedimentation of protein precipitates in salting-out process.

The effect of the composition of the partitioning system (alcohol/salt ratio) on the protein separation was also studied on model systems recently [6]. The composition range of the *tert*-butanol/ammonium sulphate/water ternary system suitable for TPP was determined. It was shown that the interfacial tension of the two-liquid system (a relevant parameter characterising the composition of the partitioning system) correlates to the amount of protein precipitated as a third phase. The higher the interfacial tension the more protein was gained from the aqueous solution. The partitioning character of the TPP was demonstrated by the results of the concentration dependence of protein separation. Furthermore, it was found that the amount of partitioned protein in the middle phase (expressed as the percentage of initial protein amount) was independent of the initial protein concentration.

There is still a need to get an insight into the mechanism of the separation process in TPP. It is supposed that detailed investigation of the middle phase, which includes analytical and mechanical characterisation, would help to get information on what processes take part in middle-phase formation. Our purpose was to determine the composition of the gel-like middle phase formed in model systems containing one single protein. The behaviour of two well-known proteins, bovine serum albumin (BSA) and ovalbumin (OVA), was compared. To reveal the role of the components and their distribution in the middle phase, the structure of the middle phase was characterised by its extension, compressibility and mechanical (elastic) properties. On the basis of the results a probable mechanism of protein separation and middle-phase formation was outlined.

Mechanical properties of biopolymer gels

Gels can be classified by the forces acting in the junction points or by the basic elements forming the network. Physical and chemical gels can be distinguished on the basis of the forces. The basic elements of the network can either be macromolecules or colloidal particles. A special type of particle gel is the protein-stabilised emulsion gel, made of protein-coated droplets aggregated in a liquid which is immiscible with the interior of the droplets [7, 8].

Various methods are available for gaining information about the structure of a gel, depending on the resolution we are interested in [9]. Spectroscopic (IR, Raman, NMR) and chiroptical (circular dichroism, optical rotation) methods can be used for the study of molecular distances (not larger than 10 nm). X-ray diffraction, neutron diffraction and light scattering are used for macromolecular distances, i.e. between 10 and 2000 nm. For less-detailed resolution, distances larger than 2000 nm, microscopic techniques (electron and optical microscopy) are used. Mechanical measurements are also useful for surveying gels in this supramolecular region.

In this work we focus on the supramolecular region: the arrangement of molecules which were examined through the mechanical behaviour of the gels.

The rheological character of gels has been widely studied. There are many theoretical [9–14] and experimental [12, 13, 15, 16] works on this subject. The basic equation used for the description of the stress–deformation behaviour of gels is the following.

The theoretical approach is based on a model with ideally elastic Gaussian chains. By considering the elasticity of one chain, the force acting between the two ends of the chain and the Brownian motion of the segments and by using physical and statistical methods the following basic equation can be deduced [12]:

$$\sigma = K_1 kT \frac{\overline{r_{cr}^2}}{r_{mp}^2} \frac{\nu}{V} \left(\lambda - \lambda^{-2}\right) \ , \qquad (1)$$

which provides connection between stress and deformation. σ is the applied force per unit undeformed area, K_1 is a constant, k is the Boltzmann factor, T is the absolute temperature,

$\overline{r_{cr}^2}$ is the mean square end-to-end distance, r_{mp}^2 is the most probable distance of the ends of the network chains, ν is the number of elastically active network chains in the volume V of the gel and λ is defined as the

extension ratio of the gel: the actual length of the gel under σ stress per initial length of the gel.

Equation (1) works well for many synthetic polymer gels, but several cases of nonideality have also been observed where this equation is not valid. For example, the purely empirical Mooney–Rivlin formula or the equation of Blatz, Sharda and Tschoegl (which can be found in the Refs [9, 12]) can be used for real gels.

The previously discussed model of rubber elasticity and the equations derived from it have been applied to biopolymer gels [12]; however, this model can be considered as a rough approximation to the behaviour of biopolymer gels. The network-forming protein is not a random coil with the exceptions of a few cases, its end-to-end distance; conformations are regulated by the secondary, tertiary and quaternary structures of the protein, which are determined by the amino acid sequence of the molecule. In addition to that the conformation of protein molecules could be changed in the presence of an interface (owing to partial unfolding), and this effect may contribute to the mechanical properties of the interfacial film. The adsorbed protein layer at the interface of the droplets dispersed in an immiscible liquid controls the stability of the emulsion and hence the structure of an emulsion gel.

Experimental

Materials

BSA fraction V (Reanal) and chicken egg OVA (Sigma) lyophilised and with purity above 98% were stored at 4 °C. 2-Methyl-2-propanol (*tert*-butanol), high-performance liquid chromatography grade, was obtained from Sigma. All other reagents were of analytical grade and each reagent was used without further purification.

Procedure

Sample preparation

Five different partitioning systems were studied; their compositions are shown in mass fraction in Table 1. The compositions of the partitioning systems are also displayed on the phase diagram of the ternary (*tert*-butanol/ammonium sulphate/water) system (Fig. 1). The two ends of the tie lines represent the composition of the immiscible upper and lower liquid phases in equilibrium. The partitioning systems indicated by numbers were chosen as follows. The interfacial tension of system 5 was 1 mNm^{-1}, that of systems 1 and 3 was 2 mNm^{-1}, while this value was 4.5 mNm^{-1} for systems 2 and 4. The systems on one tie line have upper and lower phases with the same composition, represented by the two ends of the tie line, only the ratio of the phases is different. The ratio of ammonium sulphate to water was chosen to be the same in partitioning systems 1 and 2 and also in systems 3, 4 and 5. The protein concentration was 3.5 gdm^{-3} in all systems, related to the volume of water. The masses of the components of the complete systems with protein are given in Table 1.

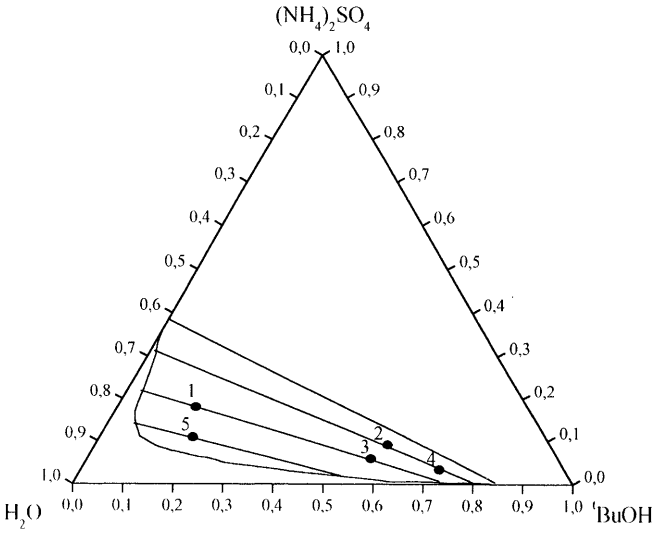

Fig. 1 The phase diagram of the *tert*-butanol/ammonium sulphate/water ternary system. The *numbers* indicate the two-phase partitioning systems chosen for investigation

Aqueous protein solution was mixed with aqueous ammonium sulphate solution in a 15-ml screw-capped, plastic centrifuge tube, and *tert*-butanol was added to the system after 1 h. The system was thoroughly mixed by turning the tubes upside down 20 times, and the mixture was centrifuged after standing for 24 h. The centrifugation was performed at 2000 rpm (672*g*) for 10 min. The system was kept at 25 °C during the whole procedure. The three-phase system obtained was used for further analysis.

Measuring the alcohol content of the middle phase by near IR spectroscopy

Reflectance near IR (NIR) spectroscopy provided an in situ method to determine the *tert*-butanol concentration of the gel.

Table 1 Compositions of partitioning systems in mass fraction. The *mass* of the components together with the protein is also given

System number	Composition of partitioning systems						
	Mass fraction			Mass (g) (with protein)			
	tert-Butanol	$(NH_4)_2SO_4$	H_2O	*tert*-Butanol	$(NH_4)_2SO_4$	H_2O	Protein
1	0.16	0.18	0.66	1.5238	1.7143	6.2857	0.0220
2	0.58	0.09	0.33	6.9048	1.0714	3.9286	0.0137
3	0.56	0.06	0.38	6.3636	0.6818	4.3182	0.0151
4	0.71	0.04	0.25	8.3241	0.4690	2.9310	0.0103
5	0.18	0.11	0.71	1.7561	1.0732	6.9268	0.0242

According to our experience liquid-phase calibration for quantitative analysis could not be used for these systems because of matrix effects. Therefore, protein gels with known *tert*-butanol/water content were prepared by a solvent-exchange procedure. The middle phases of six parallel systems were equilibrated with six different mixtures of *tert*-butanol and water for 2 h. The soaking liquid was changed at least four times during the procedure. The state of equilibrium was indicated by the constancy of the composition of the soaking liquid checked by density measurements. These gels were used for calibration. The whole calibration was carried out both in the cases of BSA and OVA gels.

The gels were measured in a standard NIR spectroscopy cuvette with a reflective sulphurous disc with a FOSS-NIR System 6250 scanning-type spectrophotometer (Pacific Scientific, USA) in reflection mode. The scanning range spread from 1100 to 2500 nm in 2-nm increments. The wavelength selected for the quantitative measurements was 1690 nm for each protein, which corresponds to the methyl group of *tert*-butanol according to Goddu and Delker [17]. Around this wavelength *tert*-butanol possesses a triple peak, which is free from the signals of water. One measurement was the average of 50 scans. The log R^{-1} transform of the spectra (R is the ratio of the reflectivity of the sample and the inner standard) was plotted as a function of the wavelength (nanometres). These spectra were then smoothed and the second derivative of these spectra were taken to resolve the overlapping bands and to eliminate the baseline shift, which are both very characteristic of NIR reflectance spectra. These transformed spectra were used in further calculations.

Determination of salt, liquid and protein content of the three different phases

The salt and liquid content of the gel can be determined by drying. The weight of the gel was measured after centrifugation, then they were heated for 24 h at 120 °C. Following the measurement of the dry weight the gels were soaked in water for at least 48 h while the water was changed frequently. Hence the water-soluble component, ammonium sulphate, was extracted from the gel. The dry weight was measured again and the protein content was obtained. All the protein denatured during heating, thus becoming insoluble in water, which was checked by the UV absorbancy of the washing liquid. The amount of electrolyte was calculated by taking the difference of the two dry weights. The liquid content was given from the initial weight and the dry material weight by subtraction.

The liquid of the gel phase was analysed following centrifugal separation at 3700 rpm (2300*g*) for 10 min.

The protein content of both liquid phases was determined using a Perkin-Elmer λ2S UV/Vis spectrophotometer. The protein concentration of samples taken from the upper and the lower liquid phases was determined by measuring the UV absorption at 280 nm. Since the upper phase did not contain protein in detectable amounts, the amount of protein in the middle phase could be calculated.

The salt content of the liquid phases was measured by drying an aliquot volume of the phases. Using the results of UV measurements the mass fraction of ammonium sulphate can be calculated by subtracting the amount of protein from the weight of the dry material. The ammonium sulphate was present in the upper phase only in traces, which did not have any effect on the density. The density of the upper and lower phases was determined using a Paar 602 density meter (A. Paar, Graz, Austria). The amount of alcohol in the system could be calculated by using the relationship between the density of a mixture and the mass fraction of *tert*-butanol [18]. The mass fraction of *tert*-butanol in the lower phase was calculated from the total amount of *tert*-butanol and the amount in the upper and middle phases.

The weight measurements were carried out with an error of $\pm 10^{-3}$ g; the weights of the samples were increased to reduce the relative error.

The determination of the composition of the phase is outlined in Fig. 2.

Studying the mechanical properties of the middle phase

The gel-like middle phase was separated from the system, and then a deformation test was applied using an apparatus designed by Horkay et al. [19]. This equipment allowed the vertical compression of the sample with measurement of deformation and stress within a wide range, from 10^{-5} to 2×10^{-3} m and from 10^{-4} to 2 N, respectively. The gel sample was placed and compressed between parallel glass plates. The samples were loaded step by step in the range 0–5 g. The deformation was measured on a scale proportional to the change in sample height, with an accuracy of 10^{-5} m. The duration of loading in each step was 10 ± 2 s. This kind of measurement provided a deformation curve, where the stress value was plotted as a function of deformation.

Information could be obtained on the reversibility of deformation by measuring the gel size when loading the sample with the smallest possible weight, 0.01 g, (which corresponds to a stress of -0.15 Nm^{-2}). The reversibility of deformation was checked twice on the sample during the whole procedure, once after reaching stress -2.2 Nm^{-2} and once after -75 Nm^{-2}.

Results and discussion

Measuring the alcohol content of the middle phase by NIR spectroscopy

Reflectance NIR spectroscopy was used to determine the amount of *tert*-butanol in the middle phase. A set of spectra measured for OVA gels used for calibration is given in Fig. 3. The spectrum labelled by 1.0 corresponds to a gel equilibrated with *tert*-butanol. The triple peak between 1670 and 1780 nm is characteristic of *tert*-butanol; 1690 nm was chosen for the calculations of alcohol content. This triple peak completely disappeared in the sample equilibrated with water (sign 0). The other spectra originated from samples containing *tert*-butanol/water mixtures in the various mass fractions given in Fig. 3. Several peaks characteristic of *tert*-butanol were

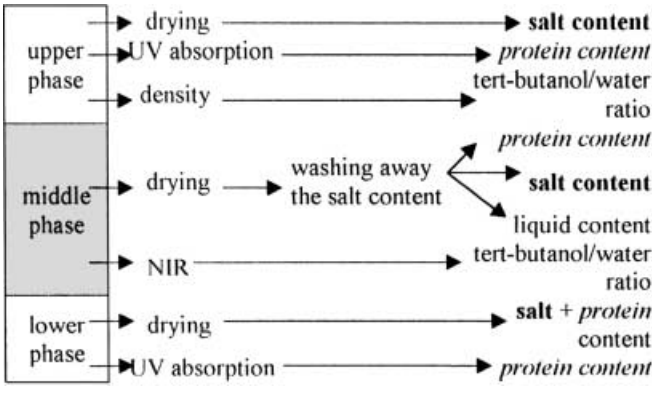

Fig. 2 Schematic outline of determination of the components

observed in these samples but with different intensities owing to the presence of water. The sample thickness and the roughness of the sample surface influence the absolute peak intensity in the reflectance NIR spectroscopy. That is why not the absolute but the relative intensity is the characteristic feature in these spectra. This fact justifies the usage of the second derivative of the spectrum, although noise was also intensified by the second derivative. The resulting error in the determination of the *tert*-butanol mass fraction was estimated as ±5–10%.

The results of the NIR spectroscopy measurements were used in the calculations of the middle-phase composition detailed in the following section (details are displayed in Tables 3 and 4).

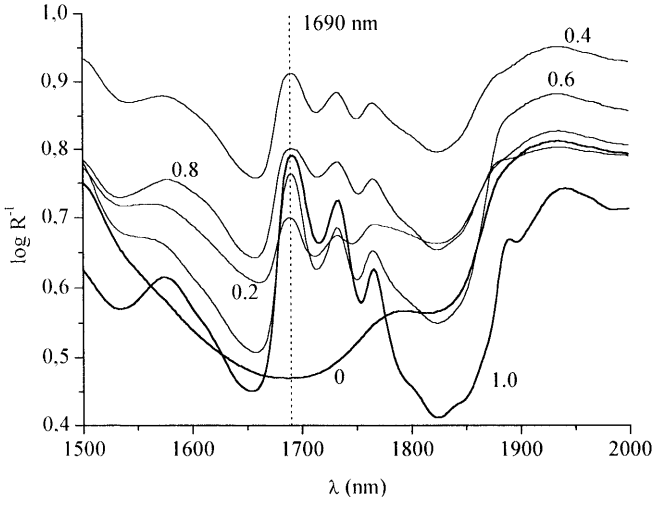

Fig. 3 The reflectance near IR spectra of ovalbumin (*OVA*) gels, equilibrated with *tert*-butanol/water mixtures of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 alcohol mass fractions. The *vertical line* shows the wavelength chosen for the quantitative analysis (1690 nm)

Determination of composition

The amount of the dry material and the liquid content of middle phases was determined in all five systems investigated for both BSA and OVA. The compositions of the middle phases in mass and in mass fraction are presented in Tables 2 and 3. The results concerning the *tert*-butanol content obtained from NIR spectroscopy measurements are also included. The number of partitioning systems (compositions given in Table 1) and the interfacial tension between the two liquid phases, γ, in the absence of protein are given in the first and second columns, respectively. The total mass of the gel-like middle phase is displayed for each system in column 3. The amount of the middle phase component in grams and in mass fraction is given in columns 4–6 and in columns 7–10, respectively.

The protein gels usually contained a large amount of liquid (80–95 wt%) even following the centrifugal compression as is seen from the data in Tables 2 and 3. The amount of protein as the network-forming material does not exceed 10 wt%. A significant difference was found between the BSA and OVA middle phases. The volume of OVA gel extended to the whole volume of the system, while that of BSA was only a fraction of the total volume. BSA coherent phases were compressed to a smaller volume by centrifugation than OVA gels. The middle phases of the BSA systems held less liquid than those of the OVA systems after the centrifugation, which could be seen from differences in the masses of these phases. As a consequence, the protein content of OVA

Table 2 The composition of middle phases in mass (*m*) and in mass fraction (*w*) for the five partitioning systems applied to bovine serum albumin (*BSA*). Abbreviations used: *sys*: system; *mph*: middle phase; *liq*: liquid, *salt*: ammonium sulphate, *but*: *tert*-butanol. γ is the interfacial tension of the protein-free partitioning system

System number	$\gamma\left(\frac{\mathrm{mN}}{\mathrm{m}}\right)$	*m*(mph) (g)	*m*(liq) (g)	*m*(salt) (g)	*m*(BSA) (g)	*w*(BSA)	*w*(salt)	*w*(but)	*w*(water)
1	2	0.252	0.211	0.025	0.016	0.06	0.10	0.49	0.35
2	4.5	0.133	0.107	0.014	0.012	0.09	0.11	0.62	0.18
3	2	0.265	0.226	0.028	0.011	0.04	0.11	0.23	0.62
4	4.5	0.204	0.173	0.022	0.009	0.04	0.11	0.62	0.23
5	1	0.263	0.242	0.013	0.008	0.03	0.05	0.48	0.44

Table 3 The composition of middle phases for the five partitioning systems applied to ovalbumin (*OVA*). Abbreviations as in Table 2

System number	$\gamma\left(\frac{\mathrm{mN}}{\mathrm{m}}\right)$	*m*(mph) (g)	*m*(liq) (g)	*m*(salt) (g)	*m*(OVA) (g)	*w*(OVA)	*w*(salt)	*w*(but)	*w*(water)
1	2	1.644	1.575	0.055	0.014	0.008	0.03	0.52	0.44
2	4.5	4.548	4.358	0.177	0.018	0.004	0.04	0.10	0.86
3	2	1.452	1.279	0.160	0.013	0.009	0.11	0.14	0.74
4	4.5	1.497	1.252	0.236	0.009	0.006	0.16	0.29	0.55
5	1	0.899	0.836	0.045	0.018	0.020	0.05	0.39	0.54

gels was below 2 wt%, while that of BSA was in the range 3–10 wt%.

There was also a difference in the salt content of the gels of the two proteins. The amount of ammonium sulphate in the OVA gels was approximately 10 times higher than in BSA gels, but the salt concentration calculated in weight percent was similar for the two proteins. It is notable that those OVA middle phases which were formed in partitioning systems of high salt content had a higher liquid and a lower salt content expressed in mass fraction (1, 2 and 5). In the case of BSA the salt content of the gels showed much variation: the average value was about 10 wt%, with one exception. Comparing the five partitioning systems, there was no significant difference in the composition of middle phases that could have been correlated to the initial composition of the system.

The most striking result was the composition of the medium of the protein gels. The components of the medium of the gel could not form a homogeneous liquid phase. These compositions (Fig. 4) are in the two-liquid-phase region of the phase diagram. That means that *tert*-butanol, salt and water are present in the gel in such a composition that leads to a heterogeneous system. This finding was valid for all but one of the partitioning systems with BSA or OVA.

The presence of two liquid phases could be deduced indirectly, since the analysis provided data on the overall content of the components of the middle phase. In order to get direct information on the state of the liquid medium, the liquid content of some protein gels was separated by centrifuging the gel alone. Two immiscible liquid phases were obtained as might be expected from the overall composition detailed previously. It was found by analysis that the composition of the liquid phases obtained from the gel corresponded to the compositions of the upper and lower liquid phases of the partitioning system. From this fact and also considering the preparation process it can be supposed that the middle phase in TPP is a concentrated emulsion stabilised by a protein network. This type of gel is called emulsion gel, which is a type of particle gel made up of emulsion droplets wrapped in surface-adsorbed protein [7, 8].

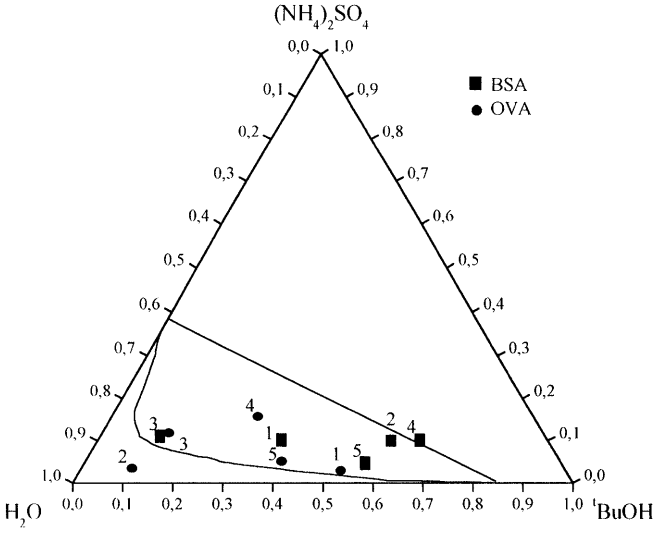

Fig. 4 The composition of the medium of the middle phases obtained in five different partitioning systems displayed on the phase diagram of *tert*-butanol/ammonium sulphate/water. The *numbers* correspond to the partitioning systems; *squares* and *circles* indicate systems containing bovine serum albumin (*BSA*) and ovalbumin (OVA), respectively

Stress–deformation behaviour of protein gels formed by TPP

Proteins were accumulated into a gel-like phase from an aqueous solution as a result of partitioning in TPP. The conditions used in TPP (standard temperature, lack of oxidising or reducing agents and the usage of the protein-friendly ammonium sulphate) permit the formation of physical connections between the network elements. The optical observations indicate that the gels are nontransparent. The mechanical properties of the gel-like BSA and OVA middle phases were investigated in order to gain information on the structure of this physical gel. The stress–deformation curves of BSA and OVA are shown in Figs. 5 and 6. The stress (σ, applied force per unit undeformed area) is plotted as a function of the extension ratio (λ, the actual height of the gel during uniaxial compression related to the initial height). The smaller the extension ratio, the smaller the

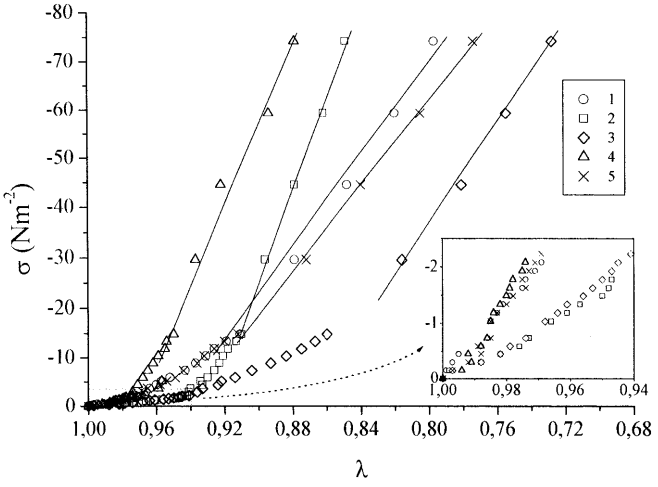

Fig. 5 The applied stress (force per unit undeformed area) as a function of the extension ratio in the case of BSA gels, obtained by applying the five partitioning systems indicated by *numbers* (detailed in Table 1). The initial section of the curves is inserted

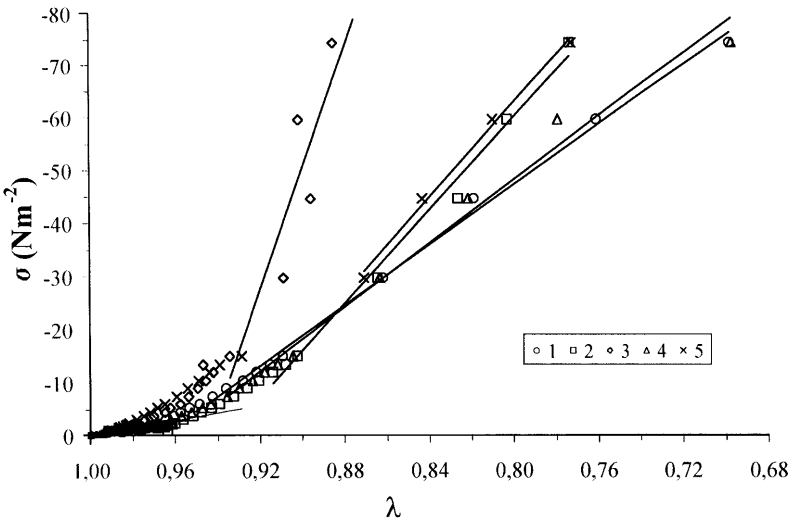

Fig. 6 The applied stress (force per unit undeformed area) as a function of the extension ratio in the case of OVA gels, obtained by applying the five partitioning systems indicated by *numbers* (detailed in Table 1)

actual height, thus the higher the deformation. The stress values are negative because the applied force was compressive. When the sample was loaded with a larger weight, the stress value was smaller.

There were two ranges of deformation where Hooke's law was valid for the gel, i.e. stress was proportional to deformation. The first region was short, started at zero deformation and lasted up to 4 or 6% (0.96 or $0.94 \leq \lambda \leq 1$). The curves of different systems ran together in this region. There was a turn in the curves: a nonlinear section was observed between stresses -2 Nm^{-2} and around -10 Nm^{-2}. The curves became linear again below -15 Nm^{-2}. In this section the curves of each protein ran separately and the slope of the curves was system-dependent.

The slope of a linear region of the curves was taken as an indicator of the gel strength. The greater the slope the harder the gel, reflecting that a large change in stress caused only a small change in deformation. It was observed that all the gels became harder during compression: the slopes of the second linear region were about 10 times higher than that of the first linear region. It was also found that the OVA gels were not so hard as the BSA gels, which can be seen from the slopes summarised in Table 4. The BSA gels were harder if they originated from a system with higher interfacial tension: systems 2 and 4 had the highest interfacial tension ($\gamma = 4.5$ mNm^{-2}) and highest slopes, while system 5 had the lowest interfacial tension ($\gamma = 1$ mNm^{-2}) and the lowest value of the slope. σ and λ were highly correlated: the correlation coefficient, R, was not lower than 0.995. In the case of OVA σ and λ were also correlated, which reflects a similar elastic behaviour as for BSA, but no correlation was found between the slopes and the relevant interfacial tensions of the systems used.

The relationship between stress and λ–λ^{-2} should be linear for macromolecular gels according to Eq. (1). Although the middle phase formed in TPP possessed a more complex structure than the gel in the model of rubber elasticity, the elastic behaviour of the gels showed a similar pattern. The actual relationship for the BSA middle phases is displayed in Fig. 7. These curves also have a break as those in Figs. 5 and 6. The initial section of the curves showed a linear connection between the stress and the deformation parameter. The final part also presented a linear correlation, but the slope of the curves was one magnitude higher than in the initial part.

Table 4 The parameters of linear regression obtained on the final section of stress–deformation curves. The *numbers* in the first column indicate the systems detailed in Table 1. σ and λ stand for the first value of stress and deformation of the final section, s is the slope of the curves and R is the correlation coefficient

System number	BSA				OVA			
	σ (Nm^{-2})	λ	s (Nm^{-2})	R	σ (Nm^{-2})	λ	s (Nm^{-2})	R
1	−8.9	0.937	462 ± 16	0.995	−7.4	0.942	284 ± 6	0.998
2	−13.4	0.913	952 ± 72	0.999	−11.9	0.914	440 ± 15	0.996
3	−29.7	0.816	510 ± 38	0.997	−14.9	0.934	740 ± 146	0.982
4	−8.9	0.962	796 ± 49	0.995	−7.4	0.936	300 ± 7	0.994
5	−14.9	0.912	431 ± 17	0.999	−29.7	0.871	452 ± 30	0.998

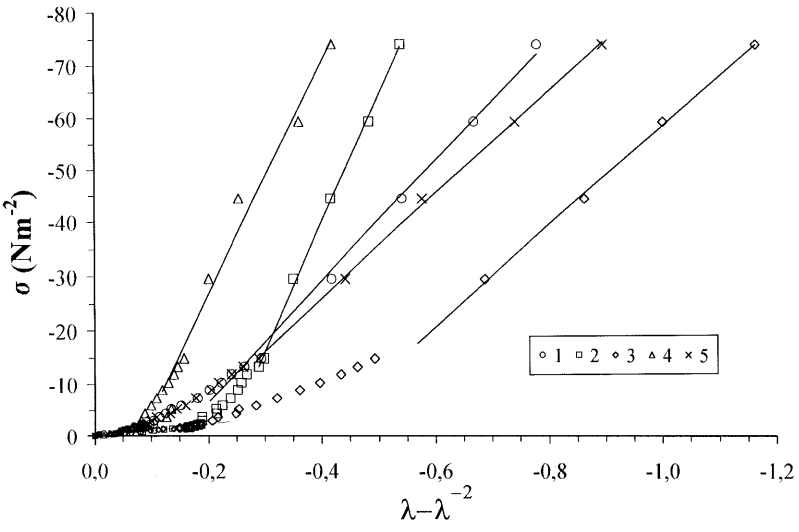

Fig. 7 The applied stress (force per unit undeformed area) as a function of the deformation parameter, $\lambda-\lambda^{-2}$, in the case of BSA middle phases, obtained by applying the five partitioning systems indicated by *numbers* (detailed in Table 1)

There was no significant change in the volume during the measurements, so the change in the front factor of Eq. (1) can be explained by the change in the concentration of the physical bonds relevant to the elastic property. This means that the large increase in the slope was due to a considerable change in the structure of the sample as a result of compression. A possible explanation could be that new interparticle interactions are formed because of the compression; the new cross-links harden the gel. Another possible explanation for the change in the elastic behaviour of the gels is based on the observations of Flemming et al. [20]. They have shown that the second linear range could be correlated to the mechanical properties, while the first region could correspond to the surface roughness and the possible nonparallelism of the sample. In our case the effect is much higher than to accept the roughness as the only cause of that kind of behaviour.

The gels were almost reversibly deformed up to -2.2 Nm^{-2} compressing stress as shown by the reversibility of deformation, which was higher than 95%. These values were slightly smaller for the OVA gels than for the BSA gels. After the change in the behaviour of the gel, which was indicated by the turn in the stress–deformation curves, the degree of reversibility slightly decreased (95–88%); this could be due to a change in the structure of the gel.

The partition of the protein in TPP takes place between the two states of the protein: the protein solvated in the aqueous phase and the protein adsorbed at the interface enlarged by emulsification [6]. It is known that conformational changes occur during the adsorption of protein at the interface [21]. The protein films are considerably expanded at the liquid–liquid interface, implying a greater extent of unfolding, which enhances the stability of the emulsion. Since OVA is more flexible than BSA, because it has fewer disulphide bonds (one instead of 17 [16, 22]), it is expected that OVA unfolds more quickly than BSA, resulting in a higher liquid holding capacity and a softer gel in TPP.

Although macromolecules were present in the system, it was an open question whether the protein gel formed during TPP was a macromolecular or a particle gel. Two different hypotheses could be considered. The middle phase could be a macromolecular gel; in this case the precipitating protein forms a network by joining the protein chains with semipermanent physical cross-links. On the other hand, the middle phase could be made of emulsion droplets wrapped in surface-adsorbed protein; in this case it is an emulsion gel, a type of particle gel. The results of the analysis of the middle phase seem to support the second hypothesis.

Conclusions

By applying ammonium sulphate and *tert*-butanol in certain amounts to an aqueous protein solution a gel-like middle phase was formed accumulating 80–90% of the total protein content of the system. The composition and the mechanical properties of that middle phase were investigated in order to get information on the process resulting in the separation of protein in a coherent phase.

The coherent phase consists of 2–10 wt% gel-forming protein and about 90 wt% or more liquid with dissolved ammonium sulphate. It was shown by NIR spectroscopy measurements and the direct analysis of the composition of each phase that the ratio of *tert*-butanol, ammonium sulphate and water in the middle phase corresponded to a heterogeneous ternary system. That means that the medium of the gel should consist of two liquid phases. The liquid separated from the gel really formed two liquid phases, with the same composition as the upper

and lower phases of the system after partitioning. These facts support the hypothesis on the formation of an emulsion gel during TPP.

As a result of the analysis of the middle phase of TPP a probable mechanism of gel formation can be described as follows. When mixing the components two immiscible liquid phases are formed, then the interfacial adsorption of the protein occurs, thus stabilising the emulsion of the two liquid phases. Thereafter leaving the systems at rest, two significant processes occur in parallel: the coalescence of emulsion droplets and the partial (or total) denaturation of the proteins, which causes the rigidity of the interfacial film and increases the barrier to the former process (coalescence). These steps result in a system composed of three phases: the middle phase is a concentrated emulsion stabilised by surface-adsorbed protein and the upper and lower liquid phases emerged from the partial breaking of the emulsion.

The mechanical investigation demonstrated the elastic behaviour of the middle phase, which is evidence for the formation of an emulsion gel phase. A well-defined increase in the hardness was found at around -2 Nm^{-2} stress, which might indicate an alteration of the network structure. The structure of the middle phase and its connection with the interfacial properties of proteins needs further investigation, probably by microscopic and other techniques.

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