SOLUBILITY AND SECONDARY STRUCTURE OF BOVINE PANCREATIC α-CHYMOTRYPSIN IN WATER–ACETONITRILE MIXTURES

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Solubility of bovine pancreatic α-chymotrypsin (CT) in water-acetonitrile mixtures was measured using UV-spectroscopy at 298 K. Water activity in mixture was varied from 0.34 to 1.0. The protein secondary structure was controlled in the same range of compositions by FTIR spectroscopy. It was found that the CT solubility depend on water activity in threshold manner. At water activity range below 0.7 the enzyme is completely insoluble and its secondary structure is close to that of air-dried protein. In the activity range from 0.75 to 0.92 solubility rises sharply. Secondary structure of CT in this range both in solution and precipitate was characterized as denatured one with predominate intermolecular β-structure due to extensive aggregation. Above $a_w = 0.92$ enzyme remains completely soluble at the protein concentration employed and its secondary structure is close to that in pure water. Comparing with the data on water state in acetonitrile at the same activity range we could explain the changes in CT solubility and structure as a result of the following factors: progressive disruption of spatial hydrogen bond network of water molecules and lowering of conformational motility of the protein molecule as water activity diminish.

Introduction

Catalytic systems, containing enzyme, organic solvent and some amount of water, are rather perspective objects of investigation both in enzymatic catalysis [1] and following such a general problem as understanding of intermolecular interactions governing the stability and functioning of proteins in unusual organic environment [2, 3].

Generally, three steps in enzymatic activity are outlined depending on water content. When water content in organic solvent is large there the same as in pure water or even higher enzyme activity is observed [4, 5]. The reduction of water content down to 70–30% (v/v) essentially affected protein properties. Enzyme molecule undergoes a denaturing conformational transition and activity reduces to zero [3, 4, 6]. Distinctive features of enzyme behaviour in media with yet lower water content (less then 30%) became increased thermostability [7, 8], ability to catalyse reactions, which do not proceed in water environment [1, 9], effect of "molecular memory" [10] and insolubility in the majority of organic solvents [11, 12]. Concerning the last, one could refer to enzyme insolubility in anhydrous organic solvents as an advantage of this system. So, being often hard in water solution to separate enzyme and reaction product, in organic solvents it is often enough to simply filter out [9]. At the same time, in spite of significant success in practical application of enzymes in organic media during last two decades, influence of organic solvent humidity on enzyme solubility were not studied systematically. There are only several examples in the literature. In particular, it was found [13] that lysozyme became insoluble in acetonitrile-water mixtures when water content in organic media decreased below 40% v/v. The solubility of proteins in essentially dry pure organic solvents was studied in details in works [11, 12]. However, even in such cases it turns out to be not possible to correlate solubility with such properties of solvents as dipole moment, dielectric permittivity and Hildebrand solubility parameter.

According to the above reasons, in the present work we attempted to investigate solubility of model enzyme bovine pancreatic α-chymotrypsin in acetonitrile–water mixtures with varying water content. Acetonitrile was chosen because it mixes up with water in any ratio and is widely used in enzymatic catalysis [4, 14, 15] and structural investigations [13, 16]. Because the solubility behaviour reflects entire interactions of protein macromolecule with an environment and can not answer whether the protein conformation is changed simultaneously, we have followed secondary structure of enzyme catalyst in the same mixtures by IR spectroscopy.

On the other hand, because of water constitutes a native media for folding and functioning of protein macromolecules, obtained results on solubility and secondary structure of enzyme were analysed in compare with those of water state in acetonitrile mixtures, obtained previously by IR spectroscopy [17, 18].

Experimental

Materials. Lyophilized bovine pancreatic α-chymotrypsin (EC 3.4.21.1, Sigma, C-4129, Type II) was used without further purification. Acetonitrile was purified and dried in accord with known recommendations [19].

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Solubility measurements. Lyophilized chymotrypsin was dissolved in distilled water. Then precalculated volumes of water protein solution and neat acetonitrile were mixed in one vial. The overall enzyme content in water-organic mixtures was kept constant throughout the range of mixture compositions and equal to 7.5×10^{-6} mol/l which corresponds to usually employed enzyme concentrations both in homogeneous and heterogeneous catalysis [4, 20]. Each of the protein containing mixtures was divided into two parts and samples were incubated at 25 °C during 1 and 24 h, respectively, followed by separation of liquid and solid phases by filtering and used for further analysis. Besides this, another two sets of experiments were carried out using 0.005 and 0.2 mol/l phosphate buffer solution instead of pure water. In all cases protein concentration in liquid phase was controlled by UV absorption measurements at 280 nm. Fraction of soluble enzyme was calculated as absorbance ratio at a given water content in acetonitrile to that in pure water.

FTIR spectroscopy. Infrared spectra were measured in Vector 22 (Bruker) FTIR device at 298 K in the range 4000–1000 cm^{-1} and resolution 4 cm^{-1}, scan number 256. Liquid phase aliquots separated from precipitate as well as water/acetonitrile mixtures of the same composition were analyzed in CaF_2 cell of 10 or 30 μm thickness regarding of water content. Precipitate samples containing also small amount of liquid phase were placed between two CaF_2 glasses and spectra were recorded from which respective liquid phase spectra were afterward subtracted. Compensation was considered to be completed when no acetonitrile absorption was detected at 2252 cm^{-1}. When needed, spectrum of pure liquid water was additionally subtracted simulated the water of hydration in protein spectra. Bulk protein content in water/acetonitrile mixtures prepared for FTIR analysis corresponded to that in solubility measurements except the range 0.92–1.0 of water activities, where protein content has been raised up to 80.0×10^{-6} mol/l for to increase its resulted concentration in solution.

Results and Discussion

Solubility in water–acetonitrile mixtures. Figure 1 presents the fraction of the soluble forms of enzyme versus thermodynamic activity of water in acetonitrile. The activity scale is used throughout for the purpose of comparison with literature data on a water state in acetonitrile and on water sorption by the protein. Relations between water mole fraction and its thermodynamic activity in acetonitrile was taken from [21]. Solubility data obtained with enzyme incubated in water–organic mixtures during 1 h was only presented because it was ascertained that increasing of incubation time up to 24 h does not influence on protein solubility within the limits of experimental error. Also using buffer with concentration of phosphate up to 0.2 mol/l instead of pure water in the mixtures did not affect the data as well.

As it can be deduced from Fig. 1, chymotrypsin solubility significantly depends on water–acetonitrile ratio and exhibits threshold shaped behavior. According to the observed solubility changes one could divide the entire range

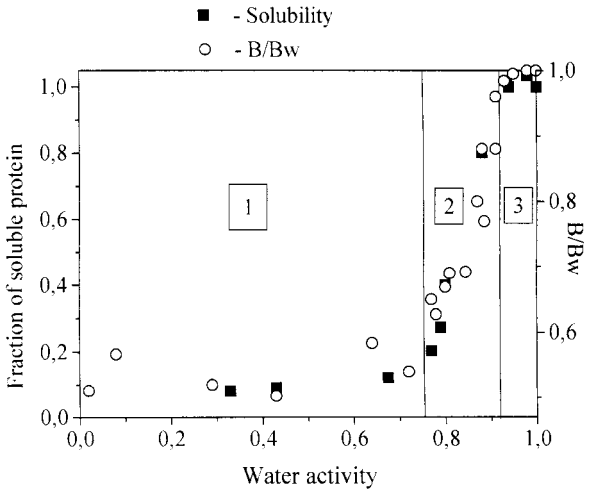

Fig. 1. Dependencies of the chymotrypsin solubility and integral extinction of water on thermodynamic water activity in acetonitrile. *Left Y-axis*, Absorbance at 280 nm normalized on protein absorbance in pure water. *Right Y-axis*, Integral extinction of water in acetonitrile mixtures normalized on the integral extinction value in pure water.

of water activities into three ranges. (1) In the range of low and medium water activity not exceeding 0.75 (water mole fraction varying from 0.01 to 0.15) the protein is completely insoluble; (2) rising activity above 0.75 sharply increases solubility which attains maximal steady value at $a_w = 0.9$ ($x_w = 0.8$); (3) In the range of upper water activities $a_w = 0.9$–1.0 ($x_w = 0.8$–1.0) the protein remains entirely soluble.

It is well known, that properties of water solutions of heterofunctional (containing hydrophobic and hydrophilic groups) nonelectrolytes are mainly determined by two factors: specific interactions between water and hydrophilic groups of nonelectrolytes and hydrophobic interactions [17, 22]. Both of them affect the hydrogen bond network of water producing changes of integral molar extinction of water (IMEW). Dependence of the relative IMEW (B/B_w) in water–acetonitrile mixtures versus water activity [17, 18] is presented in Fig. 1. As it can be seen from the figure, IMEW in the range of low and middle water activity below 0.75 exhibits nearly constant values. It was established in Ref. [22] basing on the analysis of water O–H stretch absorbance band that in this range of concentrations water exists in the form of complexes with organic molecules. Above the activity 0.75 sharp increase of B/B_w value is observed approaching that of pure water. Bearing in mind that the principal feature of pure water is existence of spatial hydrogen bond network, one could explain the above sharp increase of extinction values as a manifestation of recovery of such three-dimensional network.

Observed correlation of the data presented in Fig. 1 allows us to deduce that enzyme solubility is mainly determined by the alterations of water conditions in organic solvent. In the range of high water activity (range 3) the protein remains soluble. We could say, it is soluble where and when the free-like self-associated water remains to exist. In the range of low water activity below 0.75 the hydrogen bond network is completely destroyed and water exists

as complexes with organic molecules bringing the protein insoluble. Intermediate situation is observed in the range 2.

Secondary structure. Chymotrypsin secondary structure were assessed through the amide I spectra. This absorption arises from peptide C=O stretching vibrations and is widely used for conformation analysis in proteins [23]. As revealed the enzyme structure as well as its solubility depends essentially on mixture composition exhibiting specific features in each of the above ranges.

At low water activities (range 1) protein structure in precipitate is recognized to be close to that of dry protein in air and slightly varied with increasing water content. Protein hydration throughout this range is accompanied by some diminishing of initial β-structure recognized as slight absorbance decrease at 1637 cm^{-1} and simultaneous increase of helicity at 1658 cm^{-1} (Fig. 2). Above $a_w = 0.5$ a weak absorption at 1623-1624 cm^{-1} additionally appears due to intermolecular aggregation [23].

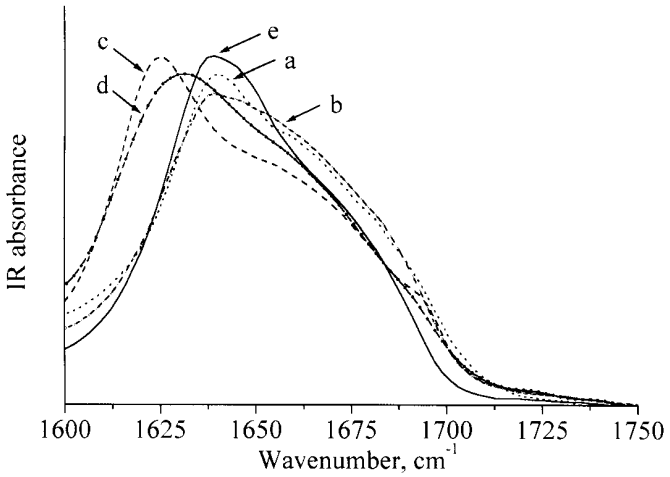

Fig. 2. IR spectra of α-chymotripsin dehydrated in air (a) and immersed in water–acetonitrile mixtures of following water activity 0.5 (b), 0.88 (c), 0.96 (d) and in pure water (e). For the aim of comparison spectra were normalized on the area of amide I band from 1700 to 1600 cm^{-1}.

In the water activity range 2 the protein is detected both in solid and liquid phases being characterized by different spectra. Exceeding $a_w = 0.75$ results in sharp increase of aggregation feature and correspondent conformation seems to become predominant (Fig. 2, $a_w = 0.9$) at the expense of another structures except that of unordered conformations absorbing at 1660–1675 cm^{-1}. Conformation of the protein solubilized in supernatant could be described as predominantly unordered with appreciable content of intermolecular aggregation (Fig. 2, $a_w = 0.9$). Observed spectral features allows us to conclude that throughout this range of water activities chymotrypsin is kept in denatured state regardless of being solid or dissolved while its conformational details may be different concerning particular phase.

In the third activity range chymotrypsin do not form a solid phase but its spectrum at $a_w = 0.96$ (Fig. 2) reveals appreciable part of aggregated structure. Nevertheless, the relative contribution from this conformation to the observed spectrum is sufficiently lower as being compared with any of 2nd range spectra. It is possible, that protein aggregation in the range 3 was promoted by relatively high protein concentration employed for properly FTIR spectra acquisition.

It is well recognized that water could act as a molecular lubricator facilitating internal dynamics of macromolecules [24, 25]. At increasing water content the conformational motility of dry lysozyme probed by ESR displays a threshold-like behavior steeply rising up above $h = 0.2$ g/g protein [25]. This hydration value is achieved at a_w above 0.75 when being referred to sorption isotherm [26] or in high water activity ranges 2 and 3 in Fig. 1. Regarding that water sorption by a number of proteins both from air and from liquid water–organic mixtures achieves nearly the same values at low and medium water activity range [27], we could suppose that the same is true also for chymotrypsin in water–acetonitrile mixtures and its conformational mobility in the range 1 has to be suppressed. This assumption is confirmed, at least qualitatively, by findings of other authors: conformational motility of protein molecules in anhydrous organic media is relatively low than in water, but essentially rises up with water content increasing [28, 29].

The presented data on secondary structure of chymotrypsin have been compared in water activity scale with internal mobility of proteins and spectroscopically obtained the water structure in acetonitrile available from literature [17, 18]. In the high activity range (3) water forms a spatial hydrogen bond network which favors hydrophobic interactions, the protein keeps its conformation native despite high level of molecular motility. Lowering water activity into the range 2 leads to progressive disruption of hydrogen bond network and consequently to weaker hydrophobic interactions. Nonpolar protein groups are brought into contact with organic solvent and provided that high conformational motility is sustained this leads to protein unfolding. At low water activities (range 1) there are no free water and no hydrophobic interactions maintaining native protein conformation. Nevertheless the enzyme molecule manifests large resistance to organic component saving most of its native structure probably due to reduced conformational motility.

We could resume, therefore, that possibly two main factors control the solubility and conformation of enzyme in water-acetonitrile mixtures, namely: (a) disruption of three dimensional water hydrogen bond network leading to weakening of hydrophobic interactions, and (b) reduction of conformational motility of protein in water-poor media.

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