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Catalytic activity, structure and stability of trypsin in an AOT-stabilised water-in-decane microemulsion

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The study of the effects of temperature and incubation time on the kinetics of hydrolysis of *N*α-benzoyl-L-arginine ethyl ester by trypsin in a water–AOT–decane reverse microemulsion revealed a good correlation between the enzymatic activity and the structural properties of the protein, as found by IR spectroscopy.

The relationship between the catalytic and structural properties of enzymes is of interest for understanding the interactions in protein–micelle complexes and the molecular mechanisms of enzyme activity in microemulsions.[1–3] However, the structure of enzymes in reverse microemulsions (organic media with immersed water droplets stabilised by a surfactant) has not been adequately studied.

The purpose of this work was to reveal the relationship between the structure and reactivity of trypsin in reverse micelles stabilised by anionic surfactant Aerosol OT (AOT).[4,5] This work is a continuation of our previous investigations into the activity of trypsin in such systems.[6]

Trypsin (EC 3.4.17.2) and *N*α-benzoyl-L-arginine ethyl ester (BAEE, a specific hydrophilic cationic substrate) were obtained from Sigma. The water-in-oil microemulsion was prepared using sodium bis(2-ethylhexyl) sulfosuccinate (AOT) from Serva as a surfactant and decane as an oil. The concentration of AOT in the microemulsion was $C_{AOT} = 0.42$ mol dm^{-3}, and the water-to-AOT molar ratio was $W_0 = 20$. The structural[7–9] and catalytic[10] properties of water–AOT–decane microemulsions were studied previously. It is also known that at $W_0 = 20$ many physicochemical characteristics of AOT-based reverse micelles, such as the hydration of AOT[11,12] and the size of micelles, remained almost unaffected by solubilised trypsin.[5] The microemulsion retained its micellar structure in the temperature range studied.[13] The enzyme concentration in the measuring cell was $E_0 = 1.1\times10^{-6}$ mol dm^{-3}, and the substrate concentration was $S_0 = 1.3\times10^{-3}$ mol dm^{-3}. The reaction kinetics was monitored by spectrophotometry at 255 nm on a Specord UV-VIS instrument. The initial reaction rate was determined[14] as $V_0 = D_{255}/\Delta\varepsilon l\Delta t$, where $\varepsilon = 700$ dm^3 mol^{-1} cm^{-1} is the differential molar absorption coefficient of the BAEE hydrolysis product in a 0.1 M Tris–HCl buffer solution (pH 8.2), and the optical path length was $l = 0.5$ cm. The structural properties of the enzyme were studied using a Vector-22 IR Fourier spectrophotometer in temperature-controlled CaF_2 cells with an optical path length of 10 μm. The protein concentration in a buffer solution or microemulsion was 5 or 2–3%, respectively. The microemulsion structure was controlled by electrical conductivity measurements using an OK-102 conductivity meter (Radelkis, Hungary) at 50 Hz and 3 kHz. The shape of the conductivity–temperature curve (Figure 1) suggests that the microemulsion retained its micellar structure in the temperature range studied.[7,9,15]

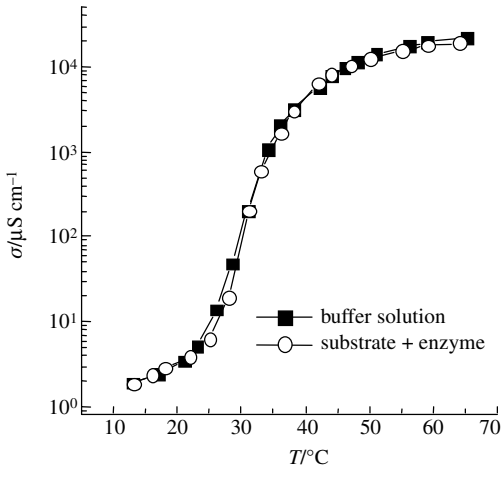

Figure 1 Electric conductivity of the microemulsion as a function of temperature.

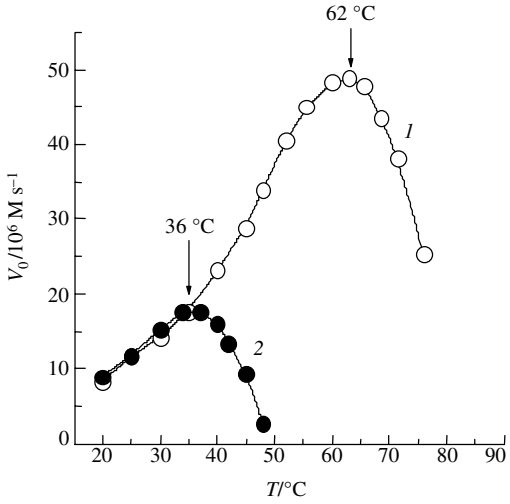

Figure 2 Initial rate of BAEE hydrolysis by trypsin as a function of temperature in (*1*) a buffer solution and (*2*) an AOT–decane–water microemulsion. Reaction was initiated by the enzyme.

Figure 2 shows the initial rate of BAEE hydrolysis by trypsin as a function of temperature in a buffer solution and in an AOT–decane–water microemulsion. The value of V_0 in the buffer solution increased as the temperature increased up to 62 °C; then, a delay was observed, which was apparently caused by enzyme denaturation. On the other hand, the rate of hydrolysis in the microemulsion increased as the temperature increased up to only 36 °C whereafter it decreased already rapidly. In both cases, the enzyme initiated the reaction of hydrolysis (the kinetics of hydrolysis was measured during 40 s). The microemulsion was kept with the substrate for 10 min at each temperature before the start of the reaction.

It cannot be excluded that an increase in the electrostatic interaction between a positively charged substrate and a negatively charged micelle surface[16] with temperature can decrease the catalytic activity of trypsin at temperatures above 36 °C. It is well known[13,17] that an increase in temperature increases the ionisation of the inner surface of micelles based on ionic surfactants. Thus, the electrostatic contact surface between the substrate and the polar heads of the surfactant increases, and the substrate is concentrated at the micelle interface, whereas the substrate concentration in an aqueous environment of the enzyme decreases.

IR spectroscopy was used to examine the effect of temperature on the molecular structure of trypsin.[18] It was found that

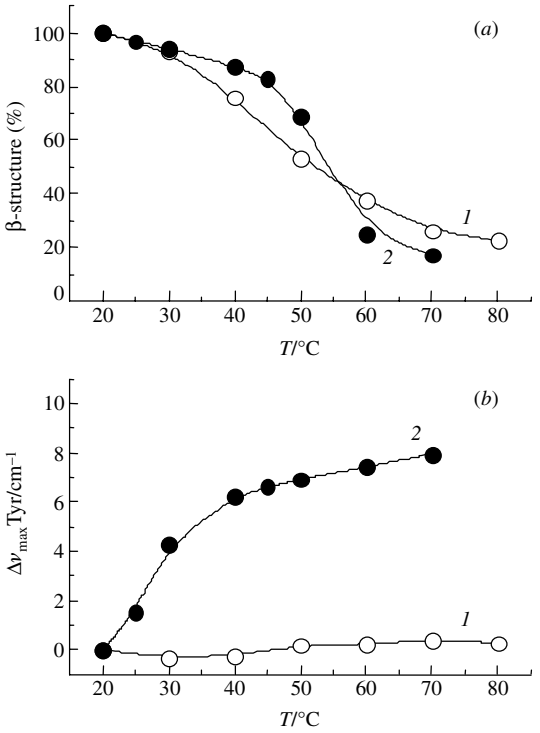

Figure 3 The temperature dependence of (*a*) the relative amount of the β-structure and (*b*) the shift of the absorption band maximum due to trypsin tyrosine residues in (*1*) a solution or (*2*) a microemulsion. Data were obtained from the second derivative of IR absorption bands at (*a*) 1630 and (*b*) 1515 cm^{-1}.

the co-operative degradation of the enzyme secondary structure (β-structure) occurs equally in the temperature range 45–60 °C in both a microemulsion and an aqueous solution [Figure 3(*a*)]. The dynamics of this process was somewhat different in the two media. As distinct from the buffer solution, in the microemulsion, the IR absorption maximum of the tyrosine residues of trypsin was shifted towards high frequencies [Figure 3(*b*)]. This shift also has the character of a co-operative transition. We assume that the behaviour of the tyrosine residue absorption band at 1515 cm^{-1} responds to changes in the enzyme microenvironment.

Although about 70% sodium counter-ions are attached to the micelle interface at 25 °C, their concentration in an aqueous phase of the microemulsion can be as high as 0.8 mol dm^{-3}.[19] It is well known that univalent cations not only are reversible competitive inhibitors of the hydrolysis of cationic substrates by trypsin[20] but also can disturb the tertiary structure of trypsin[21]

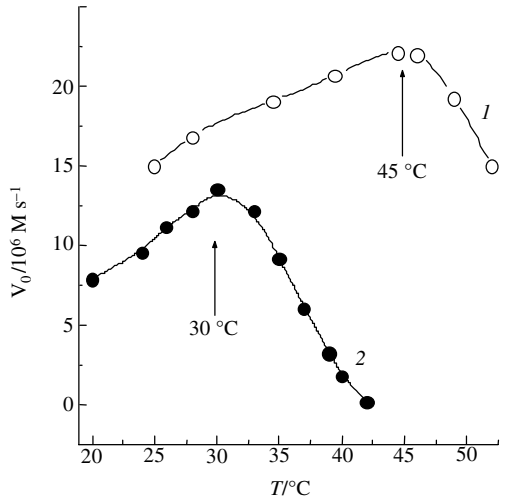

Figure 4 Initial rate of BAEE hydrolysis by trypsin as a function of temperature in (*1*) a buffer solution or (*2*) a microemulsion. Reaction was initiated by the substrate.

at a Na^+ concentration of 0.3–3 mol dm^{-3}. An increase in the temperature results in the enhanced ionisation of the micelle surface[13,17] and in the increased ionic strength of the enzyme microenvironment. It is likely that the reaction may be inhibited by the degradation of the tertiary structure of trypsin as a result of the decomposition of intramolecular ionic pairs by Na^+ ions.[22]

Reactions similar to that studied in this work are usually initiated by the insertion of an enzyme into the reaction medium. However, the reaction rate in our experiments was measured for a few tens of seconds. In structural studies, a sample was kept in the temperature-controlled cell of the IR spectrometer for 10 min at each temperature. We changed the experimental procedure now and incubated first the enzyme in the microemulsion or in the buffer solution for 10 min at each temperature and activated then the reaction by the substrate.

In both the microemulsion and the buffer solution, the maximum of V_0 (Figure 4) was now shifted towards lower temperatures in comparison with data shown in Figure 2. The absolute values of V_0 became distinctly smaller. We can state that at temperatures of about 40 °C trypsin retains still high catalytic activity in the buffer solution where it keeps about 60% of the native β-structure [Figure 3(*a*)]. In our opinion, these data indicate that in the enzyme-activated test reaction the secondary structure of trypsin cannot respond to the action of temperature, and an increase in V_0 is caused mainly by the chemical stage of the reaction. A drift of the enzyme activity maximum towards lower temperatures in the microemulsion in comparison with the buffer solution (Figure 4) correlates with temperature changes in the shift of the absorption maximum due to trypsin tyrosine residues [Figure 3(*b*)]. In our opinion, this behaviour provides support for the above hypothesis on temperature-induced changes in an enzyme microenvironment in microemulsions.

A further increase in the enzyme incubation time in the microemulsion leads to a further decrease in its activity [Figure 5(*a*)]. We experimentally found that the activity of trypsin in the buffer solution at 20 °C remained constant for 10 h. Thus, the autolysis did not take place in our experimental conditions. The secondary structure of trypsin underwent degradation in a microemulsion medium [Figure 5(*b*)]; however, its concentration was not lower than 60% over the studied time interval. The absorption maxi-

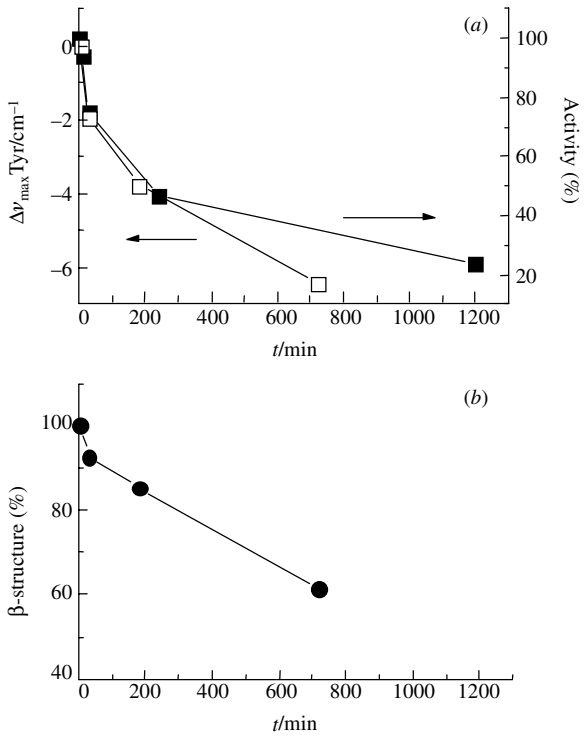

Figure 5 (*a*) (*1*) The activity of trypsin, (*2*) the shift of the absorption band maximum due to tyrosine residues and (*b*) the amount of the secondary β-structure as functions of the incubation time of trypsin in the microemulsion at 20 °C.

mum of trypsin tyrosine residues was shifted. Obviously, trypsin underwent considerable structural changes during the presence in the microemulsion for a long time.

Thus, changes in the enzymatic activity of trypsin in reverse micelles affected by temperature and incubation time resulted from structural changes in the protein, in particular, induced by changes in its microenvironment. This should be taken into account when microemulsions are used in biotechnology, pharmacy and other applications of micellar enzymology.

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