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Influence of the Microwave Irradiation on the Enzymatic Activity of Trypsin in the Presence of Poly(*N*-vinyl amides)

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Abstract—The features of the microwave irradiation effect on trypsin state and changes jn its enzymatic activity in the presence of poly(*N*-vinyl amides) at various temperatures have been studied. Comparison of the denaturizing effect of the irradiation and of convective heating has revealed, along with the thermal factors, the specific effects of microwave radiation. These effects have been found to affect both protein and polymer molecules, to change their interaction parameters, and thus, to modulate trypsin enzymatic function.

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The major aspects of the influence of electromagnetic irradiation on the physicochemical processes in solutions and heterogeneous systems have been analyzed taking various reactions as examples, including organic synthesis, complex formation, oxidation, and dissolution of organic and inorganic compounds, etc. [1–4]. Microwave radiation, by interacting with polar groups, leads to fast and uniform heating of the reactive mixture, which makes it more effective in modulating the processes parameters as compared with the traditional convective heating. On the other hand, in addition to the direct thermal effect, other types of specific influence of radiation on the system, connected with the molecules dipole polarization and leading to the structure and reactivity modulation, are possible. We have recently demonstrated [5–10] that the microwave irradiation not only accelerates the reactions of polymers compared with the thermal heating, but can even change the reaction direction.

The microwave heating is different from the traditional heating due to selectivity of energy absorption by various components of the reactive system (or by different groups of the same molecule in case of polymeric reactant).

We consider proteins to be interesting objects to investigate the special features of microwave irradiation effect on the chemical processes. The dipole moment of amino acid molecules is high, and the dielectric constant of their solutions is high as well. As the elements of proteins native conformation partially preserve the freedom of chains movement, it can be suggested that the microwave irradiation may influence the protein structure and its biological activity. The topicality of such study is also supported by the increase in the number of artificial sources of electromagnetic radiation, whereas the problem of the electromagnetic radiation influence on the biological objects is still an open question.

In the publication series [11–14], the proteins enzymatic activity as function of thermal heating and the effect of various polymers on the enzyme activity have been studied in detail. In particular, it has been demonstrated that poly(vinyl caprolactam) changes its phase state in the range of temperatures at which the enzymatic activity of trypsin is preserved; thus application of this polymer may preserve the enzymatic activity of trypsin under severe conditions.

Consequently, this work aimed at the study of special features of the microwave irradiation as affecting trypsin enzymatic activity in the presence of poly (*N*-vinyl amides) under various temperature conditions, and at comparison of the microwave irradiation effect with that of the convective treatment.

Studies of the microwave irradiation effect on the activity of trypsin revealed that crystal (powder) enzyme lost up to 15–20% of the initial activity after irradiation (800 W, 10 min). At lower radiation power (300 W, 10 min) the activity loss was lower (5–10%). Thermal treatment (90–95°C, 10 min) had practically no influence on the dry trypsin activity. The effects were different in case of trypsin solutions. As compared with the convective heating, the microwave irradiation of the buffer aqueous solution of trypsin at 50–55°C slightly increased the enzyme activity (by 5–10%), however, above 60°C the denaturation rate of trypsin upon microwave irradiation sharply increased and was higher than that at thermal heating (Fig. 1).

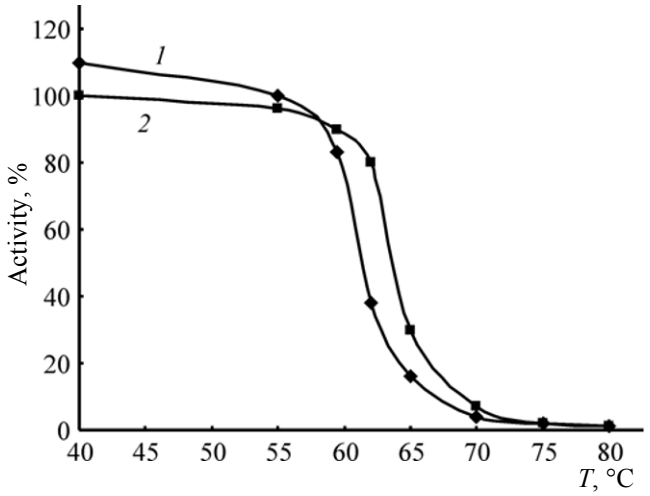

Fig. 1. Comparison of (*1*) the microwave irradiation and (*2*) thermal heating effect on the activity of trypsin in the aqueous buffer.

The experimental data showed that the effects of microwave and thermal heating on the enzymatic activity of trypsin were different. As the temperature regime was practically the same in that experiments, the difference should be due to specific features of the microwave irradiation.

The thermal stability of the enzyme was not sufficient to retain the enzymatic activity at elevated temperature. It was shown [13] that introduction of some polymers can help to preserve the enzymatic activity of proteins in wider temperature range.

Poly(vinyl caprolactam) and poly(vinyl pyrrolidone) were used in this work, they were chosen to investigate the influence of similar molecular weight polymers on the enzymatic activity under various conditions. Being of similar chemical nature, the polymers had some differences as well [11]. For instance, heating of aqueous poly(vinyl caprolactam) above 32°C (phase separation temperature T_{ph} or lower critical mixing temperature T_{cr}) lead to polymer precipitation. At the same time, water remained a good solvent for poly(vinyl pyrrolidone) up to 170°C. Thus, the side group structure significantly influenced the temperature dependence of polymer solubility [11]. This was due to differences in the hydrophilic-hydrophobic balance and dielectric constant of polymer molecules [15] that can in turn affect the efficiency of the polymers interaction with the microwave radiation. Indeed, in this work we demonstrated that under the same irradiation conditions (100 W, 5 min, 50 ml) temperature of aqueous poly(vinyl pyrrolidone) was by 1.5–2.0°C higher than that of poly(vinyl caprolactam).

At room temperature, the activity of trypsin in the presence of 4.0% of poly(vinyl caprolactam) was found to increase by 20–30% as compared with the protein in polymer-free buffer (Fig. 2). Presence of the same concentration of poly(vinyl pyrrolidone) practically had no effect on the enzymatic activity (it was increased by 3–7%). This could be due to complex formation of trypsin with the polymers. In [11], the interaction of poly(*N*-vinyl amides) with various compounds, including proteins, was studied. It was shown that the complex formation constant was higher in the case of poly(vinyl caprolactam) than in the case of poly(vinyl pyrrolidone). The addition of poly(vinyl caprolactam) to the solutions of fluorescent-labeled protein leads to the increase in the fluorescence quantum yield, and the band maximum shifted to the longwave range by 5 nm. However, upon addition of poly(vinyl caprolactam) to the free label solution, the blue shift of the fluorescence band maximum was observed: the band maximum of the free label appeared at 510 nm, while for the poly(vinyl caprolactam)-bound label the maximum was observed at 470 nm, and poly(vinyl pyrrolidone)-bound label showed the maximum at 480 nm. In this work we demonstrated that introduction of poly(vinyl caprolactam) to aqueous trypsin did not influence its UV spectrum: the band shift to the short-wave range was 2 nm. The complexing effect of the polymers was better revealed in UV spectra of *p*-nitroaniline, the major product of trypsin-catalyzed reaction. In the absence of any polymer, the absorption maximum of *p*-nitroaniline aqueous solution was observed at 381 nm, while in the presence of polymers the band

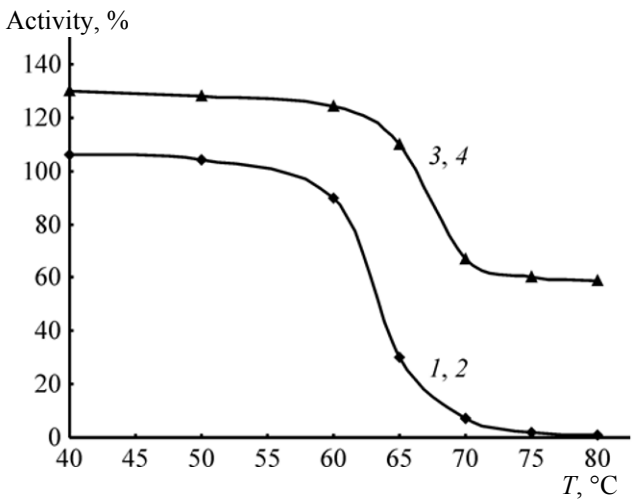

Fig. 2. Comparison of the enzymatic activity of trypsin in the buffer in presence of (*1, 2*) poly(vinyl pyrrolidone) and (*3, 4*) poly(vinyl caprolactam) upon (*1, 3*) thermal heating and (*2*, *4*) microwave irradiation. Polymers concentration was 4%.

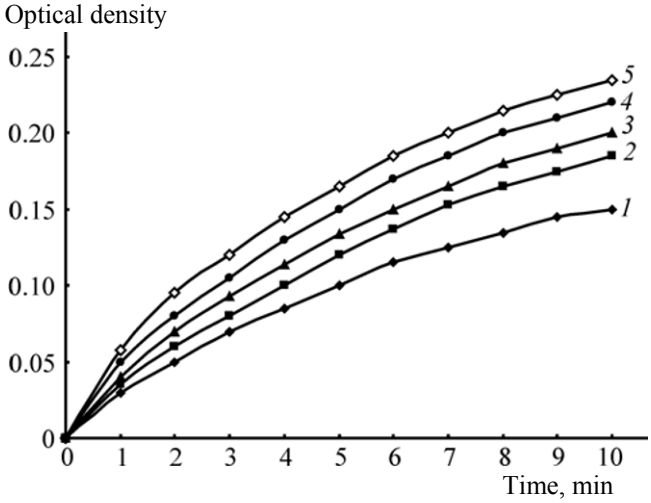

Fig. 3. Change of the enzymatic activity of trypsin upon microwave irradiation (*1*) in the aqueous buffer, in the presence of 4% of (*2*) poly(vinyl pyrrolidone) and (*3*) 8% of poly(vinyl pyrrolidone), (*4*) 1% of poly(vinyl caprolactam) and (*5*) 4% of poly(vinyl caprolactam) at (*1–3*) 65°C and (*4*, *5*) 80°C.

maximum was shifted: to 386 nm with poly(vinyl caprolactam) and to 382 with poly(vinyl pyrrolidone).

Possibly, it was the ability of trypsin to form complexes with the polymers, especially with poly(vinyl caprolactam) that lead to the increase of trypsin enzymatic activity (Fig. 2).

Upon thermal heating, the activity of trypsin decreased (Figs. 2, 3): in the presence of poly(vinyl pyrrolidone) the decrease was the same as in the polymer-free buffer medium, in the presence of poly(vinyl caprolactam) trypsin denaturated above 55–60°C, however, its activity decreased by only 40–45% (at 65–70°C). Polymers concentration also affected the observed effects. With decreasing polymer concentration in the buffer, the residual enzyme activity decreased (Fig. 3); taking poly(vinyl caprolactam) as an example, it was shown that the molecular weight change in the range of 1×10^6–1×10^5 had practically no influence on the enzymatic activity, other conditions being the same. Thus, the major factor altering the thermal stability of trypsin was the ability of poly(vinyl caprolactam), but not poly(vinyl pyrrolidone), to capture the proteins molecules near T_{cr}, thus reducing the protein chains mobility and preventing its thermal denaturation.

Upon microwave irradiation of trypsin buffer solution containing polymers, similarly to the case of thermal heating, only addition of poly(vinyl caprolactam) could retain the enzymatic activity at heating. In contrast to the polymer-free aqueous buffer solution, in the presence of poly(vinyl caprolactam) the temperature dependences of enzymatic activity were comparable for the cases of thermal and microwave heating (Fig. 2). It might be suggested that the different behavior of the polymer-free trypsin solutions upon microwave and convective heating was due to specific protein–radiation interaction; and similar interaction was possible between the microwave radiation and poly(vinyl caprolactam) molecules.

We studied the influence of microwave irradiation and of thermal heating on poly(vinyl caprolactam) molecules in detail. To do so, the IR spectra were recorded for the films of poly(vinyl caprolactam) prepared under varied conditions: at room temperature (I), under thermal heating up to 40–45°C, 48 h (II) and 85–90°C, 10 h (III), and under microwave irradiation (100–450 W, 30 min) (IV). In the last case, the solution temperature was up to 85–90°C. The comparison of films spectra showed that the sharpest distinction existed between the films I and IV. For example, the ratio of intensities of asymmetrical (2925 cm^{-1}) and symmetrical (2855 cm^{-1}) stretching vibrations bands of methylene groups decreased 1.5 times for in the film IV as compared with the film I. The same decrease was observed for the intensity of amide carbonyl vibrations (1639 cm^{-1}), simultaneously, the band width (h/2) increased 1.5 times. The most distinct changes

Relative integral intensity (ΔS) of poly(vinyl caprolactam) absorption bands[a]

Film	$\Delta S = S(\gamma_{OH})/S(\gamma_{CH})$	$\Delta S = S(\gamma_{OH})/S(\delta_{CH})$	$\Delta S = S(\gamma_{C=O})/S(\gamma_{CH})$	$\Delta S = S(\gamma_{C=O})/S(\delta_{CH})$
Original	2.3	5.7	1.0	2.4
		3.9	0.9	
Heating, 90°C	1.7			2.1
Microwave irradiation, 90°C	2.0	4.1	1.4	2.8

were observed in the range of the bending vibrations of methylene groups (1480–1420 cm^{-1}), especially in the case of the active methylene group conjugated with the carbonyl group (1420 cm^{-1}): upon microwave irradiation the intensity of this band increased 1.5–2.0 times, whereas in the case of thermal heating (90°C) the increase was much less, only 1.1–1.3 times as compared with the initial film, other conditions being the same.

On the other hand, it is known [16, 17], that water may play a great role in the conformational changes of poly(vinyl caprolactam) macromolecules. For poly (vinyl pyrrolidone) it was shown [18] that the IR spectrum of the film changed significantly upon water sorption. With water content increasing, the integral intensity of the C=O band in the poly(vinyl pyrrolidone) spectrum increased. Simultaneously, the intensity and the position of the bands at 2800–3000 and 1400–1500 cm^{-1} (stretching and bending vibrations bands, respectively, of the methylene groups) changed. It was noted that upon hydration of the film the intensity of the carbonyl-adjacent methylene group band (1424 cm^{-1}) decreased. Thus, the changes in the poly(vinyl caprolactam) film spectra may be due to the residual water. Indeed, in all the spectra the absorption at 3500–3300 cm^{-1} (stretching vibrations of OH groups) was observed, its intensity increasing in the series (III) ≤ (IV) << (II) ≤ (I).

In order to compare the spectra of the films prepared under different conditions and to reveal the influence of water on the poly(vinyl caprolactam) spectra, the relative integral intensity (ΔS) of the characteristic polymer bands was measured. From the tabulated data it followed that after the thermal heating (90°C), when water concentration in the film decreased, the integral intensity of the C=O band also decreased, being in line with the literature data [18]. After the microwave heating, the concentration of water also decreased, however, in this case the integral intensity of C=O signal was noticeably higher than that for the initial film. Just in the case of the microwave heating, the bending vibrations part of the spectrum changed the most: with water concentration in the films III and IV being almost the same, the band corresponding to the carbonyl-conjugated methylene vibrations (1420 cm^{-1}) was much more intensive in the case of microwave heating. Thus, in this case the specific effect of the microwave irradiation but not residual water concentration seemed to be the major factor influencing the spectrum features and, consequently, the conformational changes of poly (vinyl caprolactam) molecules.

It should be noted that the microwave irradiation power played significant role in the films formation. At high irradiation power (300–450 W), when temperature increased steeply to the peak value (85–90°C) within seconds, the film spectrum was similar to that of the film produced by thermal heating (90°C). At lower power (100 W) or with external cooling, temperature grew slower, within 5–10 min, and the 1420 cm^{-1} band intensity increase was the highest, namely, in this case the conditions favored the conformational changes of the macromolecule upon microwave irradiation.

Basing on the experimental observations, it might be suggested that the formation of the polymer conformational structure upon convective heating and under microwave irradiation occurred via different mechanisms. The convective heating seemed to affect uniformly all the macromolecule groups, whereas microwave heating affected mainly the polar groups. This might explain the differences in the complex formation with the enzyme, which in turn determined the protein conformation and activity.

Thus, comparison of the denaturizing effect of the microwave irradiation and that of the convective heating using trypsin as an example pointed at the existence of specific microwave-induced effects, along with the thermal factors. The specific effects were observed both in the presence and in the absence of the poly(vinyl caprolactam). This allowed a conclusion that the specific nature of microwave effects originates

from interaction of the radiation with both polymer and protein molecules.

EXPERIMENTAL

Poly(vinyl caprolactam) with molecular weight of 1×10^5 and 1×10^6 and poly(vinyl pyrrolidone) with molecular weight of 1×10^6 were used as sample poly (*N*-vinyl amides) in this work. The trypsin activity was determined using its reaction with a substrate, *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA). Upon hydrolysis of the amide bond *p*-nitroaniline was formed, its concentration was determined by spectrophotometry. The reaction was carried out in the TRIS-HCl buffer medium at pH = 8 and trypsin and substrate concentrations of 0.1 and 0.05 mg ml^{-1}, respectively (λ 400 nm, ε 10000 mol^{-1} l cm^{-1}). Pure aqueous buffer as well as that containing polymers were used. The initial rate of enzymatic reaction was determined from the slope of the linear part of *p*-nitroaniline accumulation during 20–40 s from the reaction start.

Electromagnetic field sources were microwave ovens: Samsung PG 83R (2.45 GHz, China), Mars-5, and Discover (2.45 GHz, CEM Corp. USA). Temperature was measured with fiber-optical sensor. Heating rate upon microwave irradiation was changed by altering the radiation power or by using the cooling mixture based on microwave-transparent dioxane cooled down to 3–5°C.

Thermal and microwave heating of the reactive mixtures was carried out with similar rates. UV spectra of the samples after microwave irradiation were recorded at room temperature.

IR spectra were recorded with IR Fourier-spectrometer EQUINOX 55 Bruker, UV spectra were recorded with Helios Alpha Local Control System (Thermo Spectronic, USA) spectrophotometer.

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