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Immobilization of Chymotrypsin on Selenium Nanoparticles

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Abstract In the present study, selenium nanoparticles (SeNPs) were prepared via selenious acid reduction with ascorbic acid in aqueous media and used for immobilization of hydrolytic enzyme chymotrypsin (CT). It was shown that CT is an effective stabilizer of SeNPs in solution at acidic pH values. CT did not stabilize SeNPs upon reaction in an alkaline range when 2-mercaptoethanol was used as reducing agent. UV- and visible spectroscopy and dynamic light scattering showed that synthesis temperature and concentration ratio of components play important role in the formation of Se-CT nanocomplexes. An increase in temperature of synthesis shifted the maximum of optical density toward longer wavelengths: 256, 270, and 285 nm for nanocomplexes obtained at temperature of 4, 20, and 37°C, respectively (for all studied components concentration ratios). Hydrodynamic radii of nanocomplexes also changed depending on the synthesis temperature and components concentration ratio. Aggregative stability of nanocomplexes with equal concentrations of Se and CT was studied in a wide range of media pH. Nanocomplexes aggregatively stable in solution were observed in the acidic pH range (2.8–3.75) and the alkaline range (7.1–10.5). The pH values at which the full phase separation in the system was observed lay in the range of 3.75–4.0 to 6.5–7.0. Increasing the concentration of protein stabilizer extended the pH intervals of the aggregatively stable state of nanocomplexes. Immobilization of CT on SeNPs do not lead to significant decrease of the enzyme activity.

Keywords enzyme immobilization, nanoparticle, nanocomplex, chymotrypsin, selenium

Introduction

Enzymes are proteins that function as catalysts for chemical reactions. Enzymes reduce the activation energy of a reaction, thus, greatly increasing the reaction rate. Enzymes are used in the chemical, food and pharmaceutical industries. Most enzymes that have industrial applications can be obtained on a large scale. However, native enzymes lack sufficient operational stability and it is difficult to recover and reuse enzymes after the reaction. For these reasons industrial applications of native enzymes are limited. To overcome such shortcomings various methods of enzyme immobilization can be used [1]. Being immobilized, enzymes become more stable and less sensitive to the changing environment. Moreover, immobilized enzymes ensures can be easily recycled, which reduces the utilization cost [2].

The ease of reuse and prolonged utilization of the enzyme as well as convenient product purification make immobilized enzymes preferred for most large-scale applications. For instance, immobilized enzymes are applied in industrial production of sugars, amino acids, and pharmaceuticals [3]. Furthermore, the immobilization techniques are used in biotechnology for diagnostics, bioaffinity chromatography, and biosensors [4, 5]. In prospect this technology can be applied in therapy, for example, in extracorporeal shunts [6]. It has been demonstrated that

immobilized enzymes have improved stability and greater lifespan compared to native enzymes, which allows them to work effectively in a larger range of environments [7-9]. The immobilization protects enzymes from the changing environment and therefore may allow them to retain activity at wider range of temperatures or pH values compared to native enzymes [9, 10].

There are three major routes for enzyme immobilization: binding to a carrier, encapsulation or entrapment method, and cross-linking between enzyme molecules. Each immobilization method has its advantages and drawbacks. Immobilization of enzymes by covalent bonds is among the most commonly used. Stable bonds between enzyme and carrier prevent enzyme leakage into the solution. Covalent immobilization methods are used when there has to be no enzyme in the reaction product. However, generally when enzyme is covalently bound to the carrier the enzyme activity is significantly reduced. Moreover, in this case enzyme and carrier can not be regenerated [7, 11].

In the entrapment method enzyme in confined in a polymeric network or a semipermeable membrane which is permeable only to the substrate and products while the enzyme is held inside [12]. This technology has various approaches such as gel [13] or fiber entrapping [14], or micro-encapsulation [15]. This approach is effective when substrate and products are small molecules able to pass through membranes or gels.

The benefit of reversible immobilization methods is that the matrix can be used repeatedly with new enzyme in the case of enzymatic activity loss. It is especially important in bioanalytical systems and when labile enzymes are immobilized [16].

Nonspecific adsorption is the simplest method of immobilization. It includes physical adsorption and ionic binding [17, 18]. Noncovalent immobilization can be reversed by changing the environmental conditions (pH, ionic strength, temperature, polarity of the solvent). When immobilized by adsorption enzyme usually retains its catalytic activity. Therefore this approach is cost-effective, but has disadvantages, for instance, enzyme can leak from the carrier if the interactions are not strong enough [11].

Considerable selectivity can be achieved using the affinity principle, but this technology often needs the covalent binding of an expensive affinity ligand to the support [19].

Using various nanostructured materials as carriers for enzyme immobilization seems perspective due to their large surface areas which leads to the increased local enzyme concentration. As a result enzyme activity per unit mass or volume raises compared to that of conventional materials [20]. Large surface area and low mass-transfer resistance of nanomaterials contribute to better interaction with the enzyme and improve the storage and utilization stability of the enzyme [2, 21].

Using NPs with their own biological activity for the enzyme immobilization offers the possibility of combining various useful biological properties into a single nanocomplex. The composition and amounts of components in such a nanocomplex can be controlled, depending on the particular needs of medicine or biotechnology. It is obvious that such investigations form a physicochemical basis for designing a new generation of multifunctional drugs having a broad spectrum of applications.

The hydrolytic enzyme CT was selected as matrix due to its widespread use in medicine, biotechnology, and food industry. SeNPs were chosen as carriers for CT.

Se is the essential trace element which has anti-oxidative and pro-oxidative effects, it functions as a part of various enzymes, contributes to the disease resistance, antitumor and immunomodulating activity. Se could suppress the growth of some kinds of tumor cells. But Se has a very narrow margin between its lowest acceptable levels of intake and its toxicity, so there is a risk to the supplement of Se element. It has been reported that SeNPs show high biological activity and less toxicity compared to the Se element. However, the SeNPs are also tend to aggregate into large clusters in aqueous solution, leading to a lower bioactivity and bioavailability [22].

The aim of this work was to study the effect of the conditions of synthesis (the amount of reducing agent and the concentration ratio of selenium to CT) and pH of the medium on the the spectral and dimensional characteristics of Se-CT nanocomplexes and to investigate intervals of the aggregative stability of Se-CT nanocomplexes over wide intervals of pH and NP–enzyme concentration ratios in order to determine terms of the immobilized enzyme use and kinetic parameters of CT immobilized on SeNPs.

Materials and methods

Materials

α-CT from bovine pancreas was purchased from AppliChem Co. and used as received without further purification. Selenious and ascorbic acids, 2-mercaptoethanol were of analytical grade and were used without further purification.

Preparation of Se-CT nanocomplexes

Se-CT nanocomplexes were obtained via selenious acid reduction with ascorbic acid in the presence of an enzyme in aqueous media with pH of 2.8 to 3.5 at different temperature (4, 20 и 37°C) and atmospheric pressure, according to the reaction (1):

$$H_2SeO_3 + 2C_6H_8O_6 \rightarrow Se + 3H_2O + 2C_6H_6O_6 \quad (1)$$

Nanocomplexes with various ratio of Se and CT concentrations were studied ($\nu = C_{Se}/C_{CT}$).

Characterization of nanocomplexes

The optical density was measured on a SPECORD M-40 spectrophotometer in 2.5 cm^3 quartz cuvettes at wavelengths of 220–900 nm. The medium pH was measured using a pH-673M millivoltmeter.

The average hydrodynamic dimensions R_h of the nanocomplexes were determined by quasi-elastic (dynamic) light scattering [23]. Dynamic light scattering was conducted on a Photocor Complex device (CJSC "Anteks," Russia) using a helium-neon laser ("Coherent") with the wavelength λ = 632.8 nm as the light source and a correlator with 288 channels. The correlation function was processed using the DynaLSprogram (Helios, Russia).

The study of the effect of pH on the aggregative stability of Se-CT nanocomplexes

First experiment. To the samples of Se-CT nanocomplexes NaOH of different concentrations was added to achieve wide range of media pH. Nanocomplexes with various Se concentration were studied, CT concentration was constant in all samples (C_{CT} = 0.01 wt %). Parameter ν ($\nu = C_{Se}/C_{CT}$) varied from 0.5 to 10.

Second experiment. NaOH of different concentrations was added to the samples of nanocomplexes with various CT concentrations and constant Se concentration (C_{Se} = 0.01 wt %; C_{CT} = 0.01, 0.05, and 0.1 wt %).

All samples were stored for 1-2 days until precipitation of some samples was observed.

The study of enzyme kinetics

To carry out experiments on enzyme kinetics 70 ml of protein substrate solution (sodium caseinate) with a concentration of 10 mg/ml were prepared. pH of the solution was changed by adding different amounts of 1N NaOH. The solutions were incubated at 37°C. From the obtained solution 2 ml were used as control samples. In the remaining 68 ml of the protein solution 3.4 ml of enzyme solution (or nanocomplex) with CT concentration C_{CT}=0.2 wt % were added. At certain time intervals 2 ml of the reaction mixture were sampled. To interrupt the reaction by denaturing the protein each sample (including the control) was mixed with 3 ml of 0.5N trichloroacetic acid. Then the samples were filtered and the optical density of the supernatant, which characterized the amount of digested protein, was measured at λ=280 nm.

Results and Discussion

The formation of the Se-CT nanocomplexes

Adding CT into reaction (1) simultaneously with the other components led to the formation of stable solutions with a reddish orange color. After the synthesis of SeNPs in the presence of CT solutions remained aggregatively stable for 6 months, and the Se did not change its modification after precipitation. This indicates that CT is an effective stabilizer of SeNPs in solution at acidic pH values when it carries a positive charge [24].

The dependence of hydrodynamic radii (R_h) of nanocomplexes on the reducing agent concentration in the reaction medium (C_{asc}) is presented in Fig. 1. The concentration of elementary Se (C_{Se}) obtained during the reaction was held constant (C_{Se}=0.1 wt %). The maximum dimension of nanocomplexes (~ 40 ± 3 nm) corresponded to the

stoichiometric ratio of the reaction components (concentration of ascorbic acid C_{asc} = 0.044 wt %). The hydrodynamic radii were reduced when concentration of the reducing agent in the redox system was increased. The minimum dimension (17 ± 2 nm) was observed at C_{asc} = 0.447 wt %.

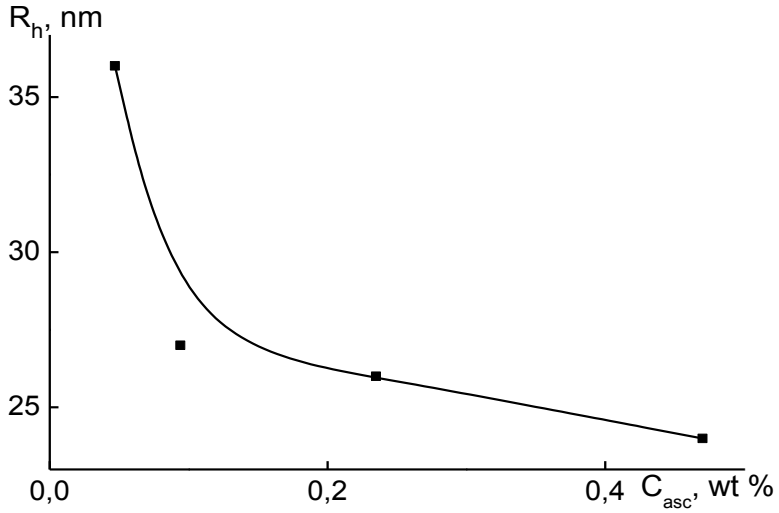

Figure 1: The effect of reducing agent (ascorbic acid) concentration on the hydrodynamic radii of Se-CT nanocomplexes

Pharmaceutical and medical applications of such nanocomplexes require their low toxicity. Most of the reports differ from each other in toxicity aspects studied. But a common feature revealed by all reports favor the postulate that small-sized NPs are more toxic compared to their large-sized counterparts [25, 26]. Further investigations were thus conducted at stoichiometric ratios of the reacting substances concentrations when hydrodynamic radii of obtained NPs $R_h \geq 30$ nm.

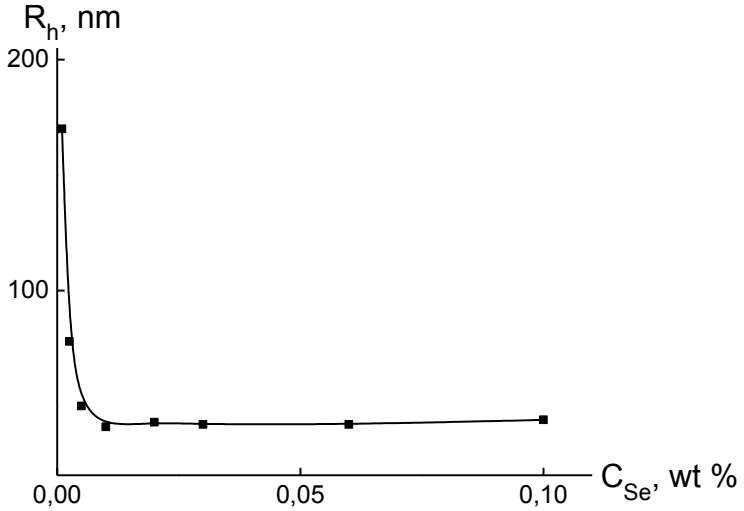

Figure 2: The effect of the Se concentration on hydrodynamic radii of Se-CT nanocomplexes at the stoichiometric ratio of the reacting substances (C_{CT} = 0.01 wt%, pH 2.5).

When the stoichiometric ratios of the reacting substances were held constant, a reduction in the absolute concentrations of selenious and ascorbic acids resulted in larger hydrodynamic radii of nanocomplexes (Fig. 2).

In the range of selenium concentrations from 0.01 to 0.00025 wt %, a notable increase in the dimension of nanocomplexes from 30 to 170 nm was observed. Increasing Se concentration from 0.01 to 0.1 wt % did not significantly change the dimensions of nanocomplexes which remained in the range of 30–40 nm. The largest NPs were thus formed at relatively low concentrations of the reacting substances [27].

An attempt was also made to synthesize nanocomplexes in an alkaline range (with initial pH 9.0–10.5) when CT is negatively charged and its conformation state is characterized by denser internal packing [28]. 2-mercaptoethanol was used as reducing agent [29]:

$$4CH_3CH_2SH + H_2SeO_3 \rightarrow Se + 2CH_3CH_2SSCH_2CH_3 \quad (2)$$

Red amorphous Se was formed as a result of reaction (2). It was immediately precipitated with no stabilization of nanocomplexes in solution was observed. Thus, CT did not stabilize SeNPs upon reaction in an alkaline range.

Stabilization mechanism and the effect of pH on the aggregative stability of Se-CT nanocomplexes

To establish the mechanism of Se-CT nanocomplex stabilization a NaCl solution of variable concentration was added to a solution of nanocomplex with ν = 1 (C_{Se}=C_{CT}=0.01 wt %). A gradually developing turbidity of solutions and the precipitation of nanocomplexes were then observed. Full phase separation (red precipitate and colorless solution) occurred when the salt concentration was 0.5 M. Adding Na_2SO_4 solution resulted in a full phase separation when salt concentration was 0.01 M, clearly demonstrating the electrostatic nature of the nanoparticle stabilization [30].

The effect of ν on the aggregative stability of nanocomplexes with constant C_{CT}=0.01 wt % at various pH values under equilibrium conditions is shown in Fig.3 (the regions of the full phase separation in the system are indicated in black, the regions of unstable behavior of the nanocomplexes – in grey, and the ranges in which the nanocomplexes produced a stable colored solution – in white).

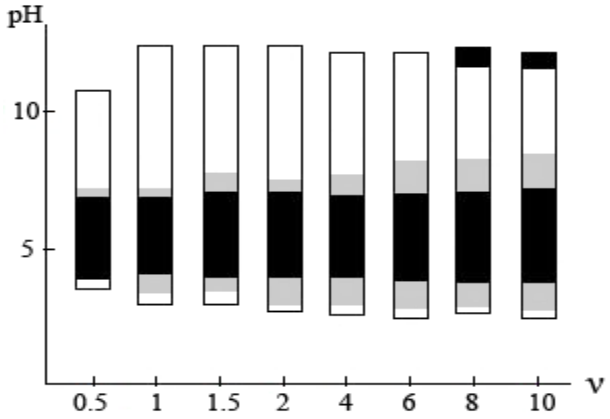

Figure 3: The effect of ν on the aggregative stability of Se-CT nanocomplexes at various pH (C_{CT} = 0.01 wt %): the regions of the full phase separation in the system (black), the regions of unstable behavior of the nanocomplexes (grey), the ranges of stable solutions (white).

Nanocomplexes aggregatively stable in solution were observed in the acidic pH range (pH 2.8–3.75) and the alkaline range (pH 7.1–10.5). The pH values at which the full phase separation in the system was observed lay in the range of 3.75–4.0 to 6.5–7.0.

It should be noted that pH intervals of the regions corresponding to full phase separation in the system were virtually independent of the concentration of Se in solution. Only at high Se concentrations (ν = 8 and 10) did another range of the full phase separation appear at strongly alkaline pH values. It may be assumed that increasing the concentration of Se to C_{Se} = 0.08 and 0.1 wt % at a constant C_{CT} concentration led to an insufficient density of CT molecule packing on nanoparticles. Hence, the interactions of nanoparticles parts not covered by CT molecules promoted their aggregation, the instability of nanocomplexes and phase separation in the system.

To determine the effect of amounts of enzyme stabilizer on the aggregative stability of Se-CT nanocomplexes similar experiments were performed while using nanocomplexes with constant Se concentration and various CT concentrations. Results are shown in Fig. 4.

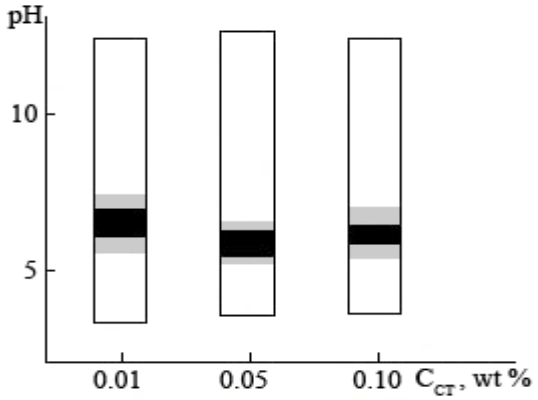

Figure 4: The effect of CT concentration on the aggregative stability of Se-CT nanocomplexes (C_{Se} = 0.01 wt %; C_{CT} = 0.01, 0.05, and 0.1 wt %): the regions of the full phase separation in the system (black), the regions of unstable behavior of the nanocomplexes (grey), the ranges of stable solutions (white).

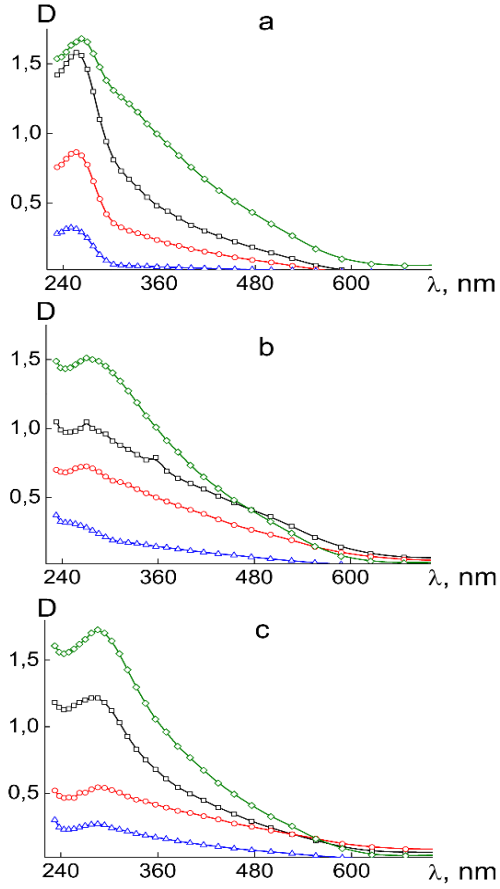

Figure 5: The absorption spectraof the Se–CT nanocomplexes: synthesis temperature is (a) 4, (b) 20, and (c) 37°C; v = 1.5 (diamonds), 1 (squares), 0.5 (circles), and0.25 (triangles).

As can be seen from Fig. 4, the intervals of phase separation in the system were reduced along with the intervals of the instable state of the system. Increasing the concentration of protein stabilizer thus extended the pH intervals of the aggregatively stable state of nanocomplexes [24].

The effect of synthesis temperature on the UV- and visible spectra of nanocomplexes

Nanocomplexes with constant CT concentration (C_{CT}=0.01 wt %) and various Se concentrations were synthesized at different temperature: 4, 20 and 37°C. For all of the investigated ν values (ν = 1.5, 1, 0.5, 0.25), an increase in temperature of synthesis shifted the maximum of optical density toward longer wavelengths. Furthermore, maxima on the absorption spectra were observed at the same wavelengths for all ν. These wavelengths were 256, 270, and 285 nm for 4, 20, and 37°C, respectively (see fig.5). It was therefore the temperature of synthesis rather than the ratio of Se and CT concentrations that was responsible for the shift of the absorption band maxima.

The effect of synthesis temperature on the hydrodynamic radii of Se–CT nanocomplexes

Measurements of the hydrodynamic radii of nanocomplexes showed that the sizes of Se-CT nanocomplexes did not exceed 100 nm (Table 1) [31].

There was a critical point (ν = 1) corresponding to the stoichiometric ratio of the components, and the character of R_h dependences on temperature and the ratio of Se and CT concentration differed strongly below and above this point.

At constant synthesis temperature increasing the amount of Se from ν = 0.25 to ν = 0.1 at all studied temperatures led to a decrease in the hydrodynamic radius. The radii passed through minima at ν = 1 (28, 33, and 38 nm at 4, 20, and 37°C respectively). This ratio of component concentrations thus corresponds to the formation of the most compact nanostructures for all of the studied temperatures. Raising ν to ν = 1.5 resulted in growth of the hydrodynamic radii.

At ν = 1, the radii of particles grew with temperature. Their sizes remained virtually unchanged at ν = 0.5, while at ν = 0.25 the hydrodynamic radii decreased significantly as temperature rose. A certain decrease in the effective dimensions with increasing temperature was also observed at ν = 1.5. Under conditions of Se deficiency (ν = 0.25) the nanostructures grew more compact as temperature rose, due apparently to a strengthening of hydrophobic interactions between CT molecules adsorbed on the same nanoparticles. Under conditions of Se excess (ν = 1.5) the change of dimensions could be due to hydrophobic interactions between Se molecules within the same nanostructure leading to compaction of the particle as a whole.

Variations in the medium's pH could be indirectly related to processes occurring in the system (Table 1). The pH value changed in the interval from 2.9 to 3.8, where CT was positively charged. A shift of pH toward the acidic side was then observed at all ν as the temperature of synthesis rose. We may assume that the dehydroascorbic acid (pK_α = 4.17) formed during synthesis dissociates more strongly as the temperature of synthesis rises. Since studied systems were most stable in acidic media [24] nanocomplexes synthesized at T = 37°C at a constant ratio of Se and CT concentrations were the most stable as well (the solutions remained stable for 6 months).

Nanocomplexes were also studied when Se and CT concentrations changed proportionally but their ratio remained constant and equal to ν = 1 (Table 2). The concentration of CT was varied from 0.1 to 0.001 wt %, and the concentration of Se changed accordingly. The largest particles of nanocomplexes were formed at insignificant concentrations of Se and CT, the hydrodynamic radii shrinking monotonically from 112 to 28 nm with a rise in the concentrations of the components. The greatest dimensions were observed at pH ~4.0 when the onset of phase separation due to particle aggregation was observed [24, 27].

Table 1: The effect of the temperature of synthesis and the ratio of Se and CT concentrations on the hydrodynamic radii of nanocomplexes

No.	ν	R_h, nm			pH		
		4°C	**20°C**	**37°C**	**4°C**	**20°C**	**37°C**
1	0.25	70	60	52	3.8	3.6	3.6
2	0.5	44	45	44	3.8	3.4	3.2
3	1	28	33	38	3.4	3.2	3.0
4	1.5	45	38	39	3.2	3.0	2.9

Table 2: Hydrodynamic radii of Se-CT nanocomplexes at ν = 1, attained in different ways (T = 20°C).

No.	C_{Se}, wt %	C_{CT}, wt %	R_h, nm	pH
1	0.001	0.001	112	4.05
2	0.005	0.005	46	3.05
3	0.01	0.01	33	2.8
4	0.05	0.05	31	2.5
5	0.1	0.1	28	2.3

The effect of pH and Se concentration on the enzyme kinetics of Se–CT nanocomplexes

Results of the study of enzyme kinetics of Se-CT nanocomplex with ν = 1 at pH 8.0 and 11.0 in comparison with the native enzyme are shown in fig. 6. As can be seen from the figure, proteolytic activity of the nanocomplex at pH 8.0 is slightly reduced compared to the activity of native CT. However, at pH 11.0 activity of the nanocomplex exceeds that of native CT.

Study of the effect of Se concentration in the nanocomplex showed that at pH 8.0 enzyme kinetics of the nanocomplexes with ν = 0.1 and ν = 1 almost coincide (fig. 7).

In the case of pH 11.0nanocomplexes with both studied ν exhibit greater activity than native CT (fig. 8). Activity of the nanocomplex with ν = 1 exceeds that of nanocomplex with ν = 0.1.

Thus, immobilization of CT on Se nanoparticles led to the shift of optimum pH of the enzyme to the alkaline pH values. The increase of the Se concentration in the nanocomplex resulted in an increased proteolytic enzyme activity.

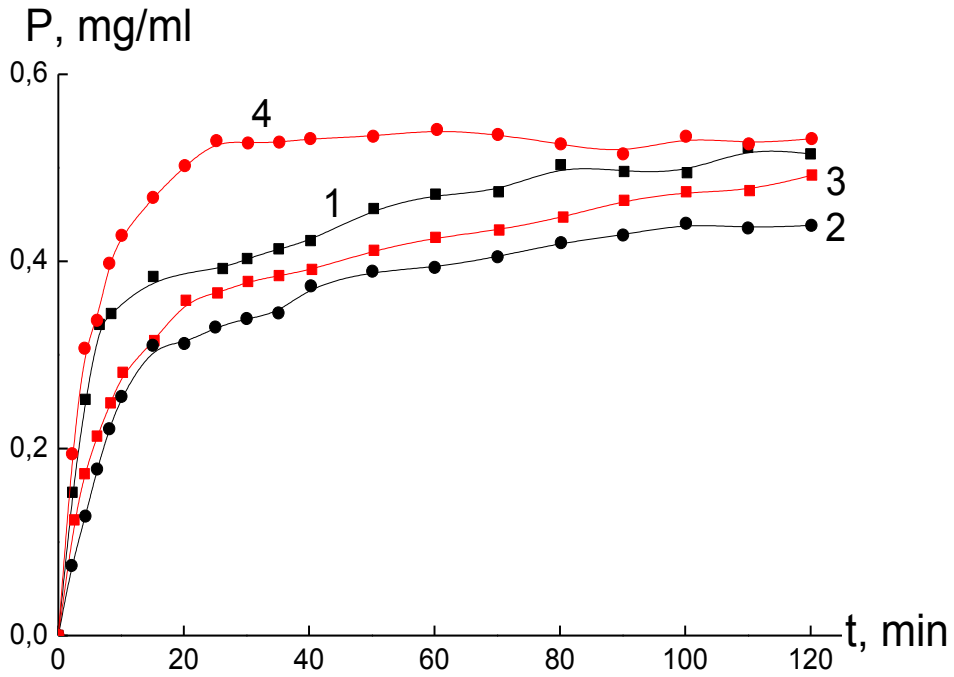

Figure 6: The time dependence of the product concentration for the enzymatic reaction of CT and Se-CT nanocomplex (ν=1) at different pH values: 1 – CT (pH 8.0); 2 – CT (pH 11.0); 3 – Se-CT (pH 8.0); 4 – Se-CT (pH 11.0).

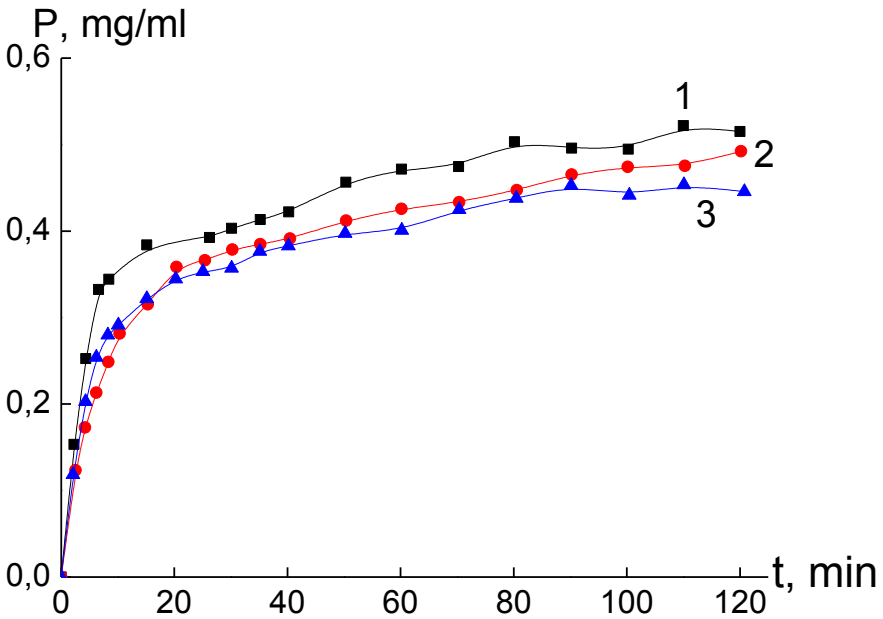

Figure 7: The time dependence of the product concentration for the enzymatic reaction of CT and Se-CT nanocomplexes at pH 8.0: 1 – CT; 2 – Se-CT (v=1); 3 – Se-CT (v=0.1).

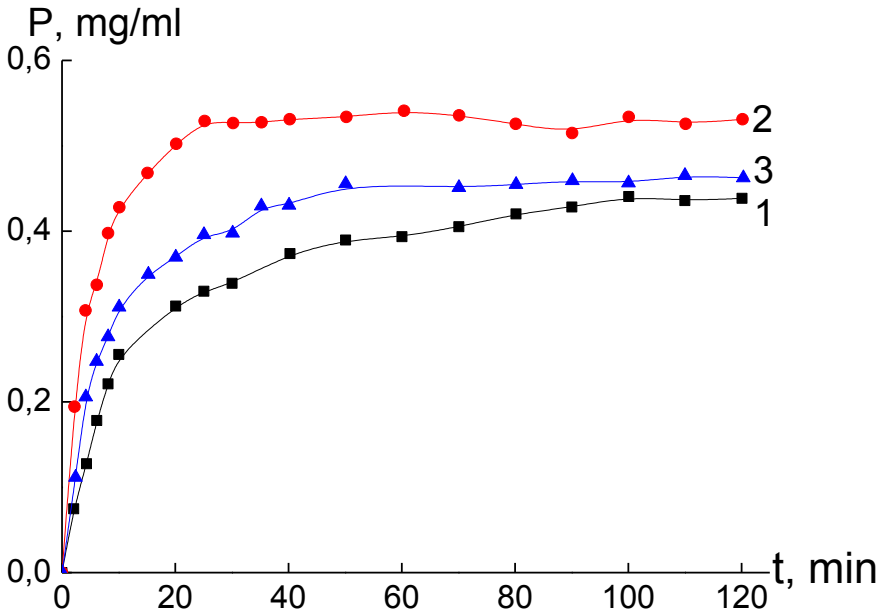

Figure 8: The time dependence of the product concentration for the enzymatic reaction of CT and Se-CT nanocomplexes at pH 11.0: 1 – CT; 2 – Se-CT (v=1); 3 – Se-CT (v=0.1)

Conclusion

In the present study the method of immobilization of CT on SeNPs was elaborated. The effect of synthesis temperature and concentration ratio of components on spectral and dimentional properties, as well as aggregative stability in the wide pH range and kinetic properties of immobilized enzyme were studied. It was shown that immobilization of the proteolytic enzyme on SeNPs is able to change the pH optimum of the enzyme. This fact can be important in the application of enzymes in medicine, biotechnology and industry.

This method can be applied to immobilization of different enzymes. The type of NPs used as carriers can also be varied in accordance with each concrete purpose of industry, medicine or biotechnology. Immobilization of enzymes on NPs provides wide opportunities of creating multifunctional bioactive nanocomplexes which combine different biological properties.

References

1. Bornscheuer, U.T. (2003). Immobilizing enzymes: how to create more suitable biocatalysts. *Angew. Chem. Int*. 42(29):3336-3337, Doi:10.1002/anie.200301664.
2. Hwang, E.T., Gu, M.B. (2013). Enzyme stabilization by nano/microsized hybrid materials. *Eng. Life Sci*, Doi:10.1002/elsc.201100225.
3. Tanaka, A., Tosa, T., Kobayashi, T. (1993). *Industrial Application of Immobilized Biocatalysts*. Marcel Dekker, New York.
4. Guibault, G.G., Kauffmann, J.M., Patriarche, G.J. (1991). In: Taylor, R.F. (editor) Protein Immobilization. Fundamentals and Applications. Marcel Dekker, New York.
5. Taylor, R.F. (1991). In: Taylor, R.F. (editor) Protein Immobilization. Fundamentals and Applications. Marcel Dekker, New York.
6. Chang, M.S. (1991). In: Taylor, R.F. (editor) Protein Immobilization. Fundamentals and Applications. Marcel Dekker, New York.
7. Spahn, C., Minteer, S.D. (2008). Enzyme immobilization in biotechnology. *Recent Patents on Engineering*, Doi:10.2174/187221208786306333.
8. Wang, Y., Hoss, U., Shah, R.W., Jenn-hann, L., Van, A., William, P., Cochran, B.B.: US20080026473 (2008).
9. Moore. C.M., Akers, N.L., Hill, A.D., Johnson, Z.C., Minteer, S.D. (2004). Improving the environment for immobilized dehydrogenase enzymes by modifying nafion with tetraalkylammonium bromides. *Biomacromolecules*, 5(4):1241-1247.
10. Schaaf, M., Heide, L.E., Eckhard, W., Leisrjer, Y.T., El-Olemy, M.M. (1993). Immobilization of Isochorismate Hydroxymutase. Comparison of Native versus Immobilized Enzyme. *J Nat Prod*, 56(8):1304-1312.
11. Brena, B., Gonzalez-Pombo, P., Batista-Viera, F. (2013). Immobilization of Enzymes: A Literature Survey. *Methods Mol Biol*, Doi:10.1007/978-1-62703-550-7_2.
12. O'Driscoll, K.F. (1976). In: Mosbach, K. (editor) Methods in enzymology. Academic, New York.
13. Bernfeld, P., Wan, J. (1963). Antigens and enzymes made insoluble by entrapping them into lattices of synthetic polymers. *Science*, 142:678–679.
14. Dinelli, D., Marconi, W., Morisi, F. (1976). In: Mosbach K (editor) Methods in enzymology. Academic, New York.
15. Wadiack, D.T., Carbonell, R.G. (1975). Kinetic behavior of microencapsulated *β*-galactosidase. *Biotechnol Bioeng*, 17:1157–1181.
16. Gupta, M., Mattiasson, B. (1992). In: Suelter, C.H. (editor) Methods of Biochemical Analysis, volume 36. Wiley, New York.
17. Messing, R.A. (1976). In: Mosbach, K. (editor) Methods in Enzymology, volume XLIV. Academic Press, New York.
18. Woodward, J. (1985). In: Woodward, J. (editor) Immobilized Cells and Enzymes: A Practical Approach. IRL, Oxford.
19. Solomon, B., Hollaander, Z., Koppel, R., Katchalski-Kazir, E. (1987) In: Mosbach, K. (editor) Methods in Enzymology, volume 135. Academic Press, London.
20. Kim, J., Grate, J.W., Wang, P. (2006). Nanostructures for enzyme stabilization. *Chem. Eng. Sci*, Doi:10.1016/j.ces.2005.05.067.
21. Kim, J.B., Grate, J.W., Wang, P. (2008). Nanobiocatalysis and its potential applications. *Trends Biotechnol*, Doi:10.1016/j.tibtech.2008.07.009.

22. Xuewei, J., Qingye, L., Siwei, Z., Xiaojuan, X., Zhang, L. (2015). Construction of selenium nanoparticles/β-glucan composites for enhancement of the antitumor activity. *Carbohydrate Polymers*, Doi:10.1016/j.carbpol.2014.09.088.
23. Brown, W. (1993). *Dynamic Light Scattering: the Method and Some Application*. Clarendon Press, Oxford.
24. Borovikova, L.N., Titova, A.V., Matveeva, N.A., Pisarev, O.A. (2013). Stabilizing Selenium Nanoparticles with Chymotrypsin: The Effect of pH and Nanoparticle–Enzyme Concentration Ratios on the Stability of Nanocomplexes. *Russ. J. Phys. Chem. A*, Doi:10.1134/S0036024413060058.
25. Sajid, M., Ilyas, M., Basheer, C., Tariq, M. et al (2015). Impact of nanoparticles on human and environment:review of toxicity factors, exposures, control strategies, and future prospects. *Environ Sci Pollut Res*, Doi:10.1007/s11356-014-3994-1.
26. Carlson, C., Hussain, S.M., Schrand, A.M. et al (2008). Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. *J Phys Chem*, Doi:10.1021/jp712087m.
27. Ershov, D.Y., Kipper, A.I., Borovikova, L.N., Pisarev, O.A. (2013). Effect of the conditions of synthesis and the pH of the medium on the dimentional characteristics of nanocomplexes of selenium with chymotrypsin. *Russ. J. Phys. Chem. A*, Doi:10.1134/S0036024413120066.
28. Lamri, R., Biltonen, R. (1969). In: Timasheff, S.N., Fasman, G.D. (editors) Structure and Stability of Biological Macromolecules. Marcel Dekker, New York.
29. Gao, X., Zhang, J., Zhang, L. (2002). Hollow Sphere Selenium Nanoparticles: Their In-vitro Anti Hydroxyl Radical Effect. *Adv. Mater*, Doi:10.1007/s001090000086.
30. Napper, D. (1984). *Polymeric Stabilization of Colloidal Dispersions*. Academic, London.
31. Borovikova, L.N., Titova, A.V., Kipper, A.I., Pisarev, O.A. (2015). Effect of the Temperature of Synthesis on the Spectral and Dimensional Characteristics of Selenium–Chymotrypsin Nanocomplexes. *Russ. J. Phys. Chem. A*, Doi:10.1134/S0036024415030115.