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Improving the thermostability of horseradish peroxidase by incorporating into water-immiscible coacervates

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Abstract

Looking for novel matrix materials for encapsulation of enzymes based on water-immiscible coacervates prepared by reaction of negatively charged hyaluronic acid and a positively charged recombinant mussel adhesive protein containing tyrosine residues was the subject of the investigation of this work. The results of experimental study of the thermostability of horseradish peroxidase (HRP) by means its encapsulation in these coacervates at temperature from 30 to 95°C is presented in this paper. The Michaelis-Menten equation was applied to analyze of the enzymatic activity of HRP. The kinetic parameters were interpreted using a Lineweaver-Burk plot. According to the data obtained, Michaelis-Menten parameters, K_M and K_{Cat}, interpreted from the Lineweaver-Burk plots, were 0.271 mM and 2265 s^{-1} for the free HRP and 0.325 mM and 2158 s^{-1} for the rMAP/HA coacervate, containing HRP, respectively, which indicate that the enzyme did not lose its activity during the coacervate formation. It was founded that the free enzyme began to lose activity above 40°C, while the encapsulated HRP remained stable to 85°C. The encapsulated HRP lost only 18% and 25% of activity at temperature of 90 and 95°C, respectively, while as free HRP loses all its initial activity, although they show similar activity at room temperature.

Key words: encapsulation, hyaluronic acid, coacervate, recombinant mussel adhesive protein.
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Introduction

The successful use of biological molecules (enzymes, antibiotics, vitamins) to many applications depends on their ability to maintain activity under conditions that are not characteristic of most enzymatic reactions, in particular, the effect of high temperatures accompanying the production of animal feed (~ 95°C), or long-term storage conditions. Enzyme immobilization on the surface of a carrier (fixation or incorporation of biomolecules into it) may address many of the issues listed above. Methods commonly employed for this purpose are covalent bonding (Grazú et al., 2005), entrapment (Yan et al., 2006) and physical adsorption (Ladero et al., 2006). Adsorption is considered as the dominant mechanism of interaction of a protein with a surface and, in principle, is the initial event that precedes immobilization through covalent bonding or encapsulation. As a result, the immobilized enzyme acquires an increased stability at high temperatures (Koutsopoulos et al., 2005). However, the physical adsorption of enzyme on most hydrophilic support is not generally strong enough (Cahyaningrum et al., 2014). The key to successfully utilizing enzymes for biotechnological applications is to ensure that

upon immobilization the enzyme remains functional. Strategies for the covalent immobilization of enzymes have been reviewed in many publications (Hirsh et al., 2010; Novick and Rozzell, 2008; Ahmad, R., Sardar, M. 2015). The covalent binding is usually very strong, and leakage of enzyme from the support is usually minimal. However, covalent immobilization of enzymes onto solid supports may lead to damage in their structures, thereby causing loss of activity (Matosevic et al., 2011). Many scientists and engineers concentrated on enzyme immobilization using nanoscale material as support by using traditional immobilization methods, mainly as covalent attachments (Datta et al., 2013; Homaei et al., 2013). As time passed, the revolutionary immobilization method was developed based on the size of the enzyme with a nanometer scale such as single enzyme nanoencapsulation and self-entrapment by silaffin (Min and Yoo, 2014).

In our opinion, more promising way to increase the enzymes' thermostability is related to different types of encapsulation despite the various post-translational machineries. Encapsulation provides a platform for protecting enzymes from thermal inactivation during prolonged exposure of increased temperatures, provided that adequate interactions between the matrix and the enzyme occur. In addition, it is necessary that the matrix materials also withstand high temperatures (Unsworth and Koutsopoulos, 2007). The main attention focuses on correlating thermostability and enzymes' activity. Various materials are used in creating matrices: silica based materials (e.g. sol-gel matrices, mesoporous silica; Pierre, 2004), aluminosilicates (Lee et al., 2005), polymers (Yan et al., 2006; Bolis et al., 2004) and organoclays (Patil et al., 2005), but they all have some limitations.

Looking for novel matrix materials, there were attracted by recent publications of (Kim et al., 2016; Choi et al., 2011) on water-immiscible coacervates prepared by reaction of negatively charged hyaluronic acid (HA) and a positively charged recombinant mussel adhesive protein (rMAP) containing tyrosine residues (rMAP/HA). The results on experimental study of thermostability improvement of horseradish peroxidase (HRP) by its encapsulation in these coacervates are presented in this article.

Materials and Methods

Horseradish peroxidase (HRP) is an enzyme that is widely used in bioassay and biosynthesis and whose properties have been studied for decades (Veitch, 2004), was chosen as a model enzyme for the present study. Among existing encapsulation methods or genetic approaches applied to HRP, the nanogel demonstrates unprecedented stability and ease of execution of the two-step encapsulation procedure. An advantage of enzyme encapsulating in the nanogel is that the second step, which is aqueous in situ polymerization, can be repeated by adding monomers and cross-linkers to react with the primary nanogel so as to give the final product that contains a single enzyme in expected size and shape. It was founded that the encapsulated enzyme (HRP) exhibits similar biocatalytic behavior with a free HRP, but significantly improved stability at high temperature (Yan et al., 2006).

Nevertheless, in our work, the coacervate which was prepared by reaction of negatively charged hyaluronic acid (HA) and a positively charged recombinant mussel adhesive protein (rMAP), was used to encapsulate the enzyme (HRP). The two starting materials are well known for their thermal stability, which in the case of HA can exceed 130°C (Gousse et al., 2012) and rMAP which is the strong and water-insoluble mussel adhesive proteins have were used to formation of a water-insoluble coacervates. Such features of mussel adhesive proteins (MAPs), as biocompatibility and strong adhesiveness explain their high potential in different applications in medicine, including design of artificial tissues. Mussels are able to keep strong adhesion in different environment to survive. For our study, a significant advantage of MAPs is their ability to attach to all types of inorganic and organic surfaces.

The potential of rMAP/HA coacervate as an effective binding material for grafts is described in study (Kim et al., 2016). The authors of article mentioned that their investigation of interaction between HA and rMAP was stimulated by the sandcastle worm-inspired complex coacervation using these two components.

A composition of foot proteins type 1 (fp-1) together with proteins type 2 (fp-2) retrieved from mussels is example of extracted MAPs during single commercially available process. The evidence of low efficiency of this process is the fact that about 10000 mussels are required to obtain 1 g of final product. The foot protein type 5 (Mgfp-5) and type 3 (Mgfp3) manufactured from Escherichia coli display advantageous adhesiveness and other attractive properties. Tyrosinase-modified recombinant fp-5 showed ~ 1.11 MPa adhesive shear strength, which is the first report of a bulk-scale adhesive force measurement for purified recombinant of natural MAP type. The complex coacervate with using recombinant fp-5 and hyaluronic acid was prepared as an efficient adhesive composition having improved bulk adhesion strength (Hwang et al., 2007; Lim et al., 2010). At the same time, MAP type 5 (fp-5) is characterized by several problems for practical applications such as low production yield from toxicity to host E. coli cellular structure, as well as limited solubility on post-purification step and related to this fact challenges in purification process.

Therefore, we used MAP of the hybrid type 151 (fp-151), which has a number of important properties, and efficiency as presented in the study of Hwang et al. (2007). Previously Kim et al., (2008) describe recombinant protein which was based on 6 repeats of the fp-1 deca-peptide fragments at both C-termini and N-termini of protein fp-5, which was designed with a high level of success and obtained in *E. coli* system and considered as potentially important natural adhesive. However, the recombinant fp-151 system also requires the limitations in quantity, as there is a need for further progress in process of separation from Gram-negative bacterium *E. coli*. Safety issues related to the presence of *E. coli* need to be considered when process of protein purification is taking place.

Thus, taking into account all the above the following two-step procedure was performed according to the method of Kim et al, (2016) and enzymatic activity assay performed following published procedure of Yan et al., (2006).

The first stage: the isolation procedure of rMAP.

Escherichia coli BL21 (DE3) cells containing the plasmid encoded by the fp-151 hybrid recombinant protein were cultured in an incubator at 37°C at 250 rpm in Luria-Bertani medium (LB) supplemented with 50 µg/ml ampicillin. When the culture density reached the optimum range of 0.7 at 600 nm (OD_{600}), $1 \cdot 10^{-3}$ M isopropyl-β-D-thiogalactopyranoside was added to induce rMAP expression. After this procedure, the culture was incubated for an additional 8 hours with stirring at 37°C. Bacteria were collected by using centrifuging at 7,500 g for 10 minutes at 4°C and the collected cell pellets resuspended in lysis buffer ($10 \cdot 10^{-3}$ M Tris-HCl and $100 \cdot 10^{-3}$ M sodium phosphate, pH = 8.0) per gram wet weight. The cells were lysed in a buffer using an OS cell-disruption system, followed by centrifugation at 15,000 g for 20 minutes at 4°C. After the centrifugation procedure, which is process of purification from impurities, the lysate was treated with Tris-Triton-EDTA buffer ($50 \cdot 10^{-3}$ M Tris-HCl, 1% Triton-X-100, $1 \cdot 10^{-3}$ M ethylenediaminetetraacetic acid and $0.1 \cdot 10^{-3}$ M phenylmethanesulfonyl fluoride, pH = 8.0), then resuspended in 25% (v/v) acetic acid for rMAP extraction. The precipitate was separated from the homogenate by centrifuging at 14,000 g for 30 min at 4°C and the supernatant was collected and then freeze-dried.

The second step: the making of rMAP/HA coacervate containing horseradish peroxidase (HRP).

To obtain the complex coacervate, each polyelectrolyte (rMAP and HA) was dissolved in PBS buffer at concentration 1 mg/ml. In addition, the obtained saturated solution of HRP in PBS was filtered through filter paper. For complex coacervation between rMAP and HA solutions, the optimal ratio of 7:3 (w/w), was used, which was proposed in the study (Kim et al., 2016). The condensed coacervate phase was prepared by stirring the mixture of prepared HRP, rMAP and HA solutions at ratio 10:7:3 for 20 min at 4°C. The brown precipitate was collected by centrifuging at 14,000 g, the supernatant was separated and the precipitate, frozen to the temperature of liquid nitrogen and freeze-dried.

The final procedure: measurement of enzymatic activity.

The biocatalytic activity was examined using 3,3',5,5'-tetramethylbenzidine (TMB) as the substrate. The incubation mixture contained 0.9 ml of 100 mM phosphate citrate (pH=5.5) containing 1.1 mM H_2O_2, 0.05 ml

0.02 M H_2O_2, and 10 μM 0.2 μg / ml HRP. The reaction was initiated by adding 0.05 ml of DMSO containing 0.02 M TMB and monitored by measuring OD_{655} using a UV-Visible Spectrometer (Shimadzu). The oxidation rate of TMB were interpreted from slope of the initial linear parts of the adsorption curve at 655 nm using a molar absorption coefficient (39000 $M^{-1}cm^{-1}$) for the oxidation product of TMB (Frenkel-Mullerad and Avnir, 2005).

Result and Discussion

To determine thermal stability of the free HRP and rMAP/HA coacervate, containing HRP, the sample was incubated at different temperature (from 30 to 95°C) at given period. Then the sample was incubated 2 hours at 4°C and room temperature sequentially before subjected to HRP activity assay.

A comparison of the thermal stability of free HRP and rMAP/HA coacervate containing HRP was performed at a temperature of 30 to 95°C at pH=7.0. The free HRP started to lose its activity above 40°C while the encapsulated HRP remained stable until 85°C. As shown in Figure 1, at temperatures of 90°C the encapsulated HRP lost only 18% activity and at 95°C - 25% activity, while the free HRP loses all its initial activity, although they show similar activity at room temperature.

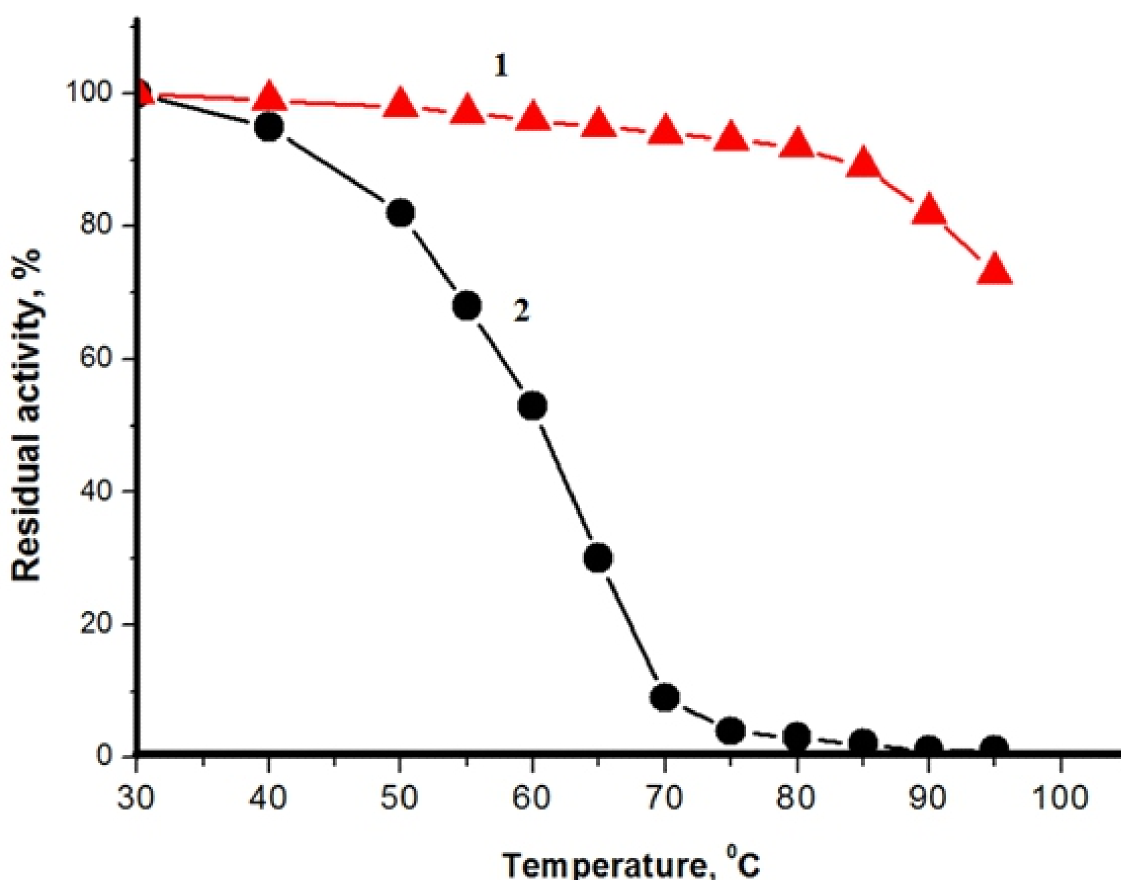

Figure 1: Thermal stability of the rMAP/HA coacervate, containing HRP (1) and free HRP (2) at pH=7.0

Also, the dependence of the activity of free HRP and encapsulated HRP on the incubation time was studied (Figure 2). As follow up from the Figure 2, the activity of the free enzyme decreases by a 50-fold during the incubation at 85°C for 60 min. The activity of the encapsulated enzyme (HRP in rMAP/HA coacervate) decreases weakly, at 1.14-fold during the incubation at 85°C. Thus, the activity of the encapsulated enzyme before the beginning of incubation is 0.032 units, after 20 min of incubation - 0.031 units, after 60 min of incubation - 0.028 units. In this case, the initial activities of the free and encapsulated enzyme practically coincide.

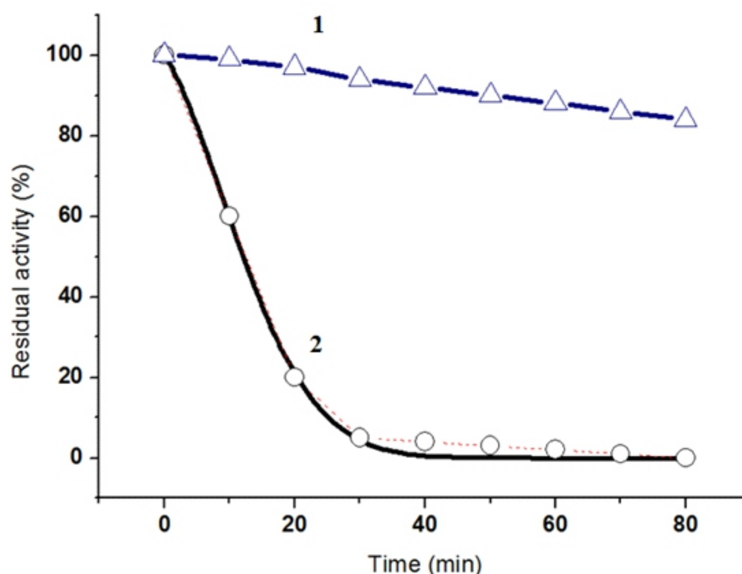

Figure 2: Thermal inactivation kinetics of the encapsulated HRP (1) and free HRP (2) at 85°C in the oxidation of TMB during the incubation time at pH = 7.

Thus, the inclusion of the enzyme in the coacervate leads to a decrease in the catalytic activity of the enzyme after incubation for 60 min at 85°C by 12%, while the free enzyme loses its activity almost completely (98%). The maintain of HRP activity in rMAP/HA coacervate to 98% can be explained by an increase in the amount of available enzyme as a result of expansion of the coacervate pores at the increase of the temperature.

The Michaelis-Menten equation was used for the analysis of enzymatic activity, which was applied to the free HRP and rMAP/HA coacervate, containing HRP with a TMB concentration of 0 to 1 mM. The kinetic parameters were interpreted using a Lineweaver-Burk plot (Veitch, 2004; Figure 3).

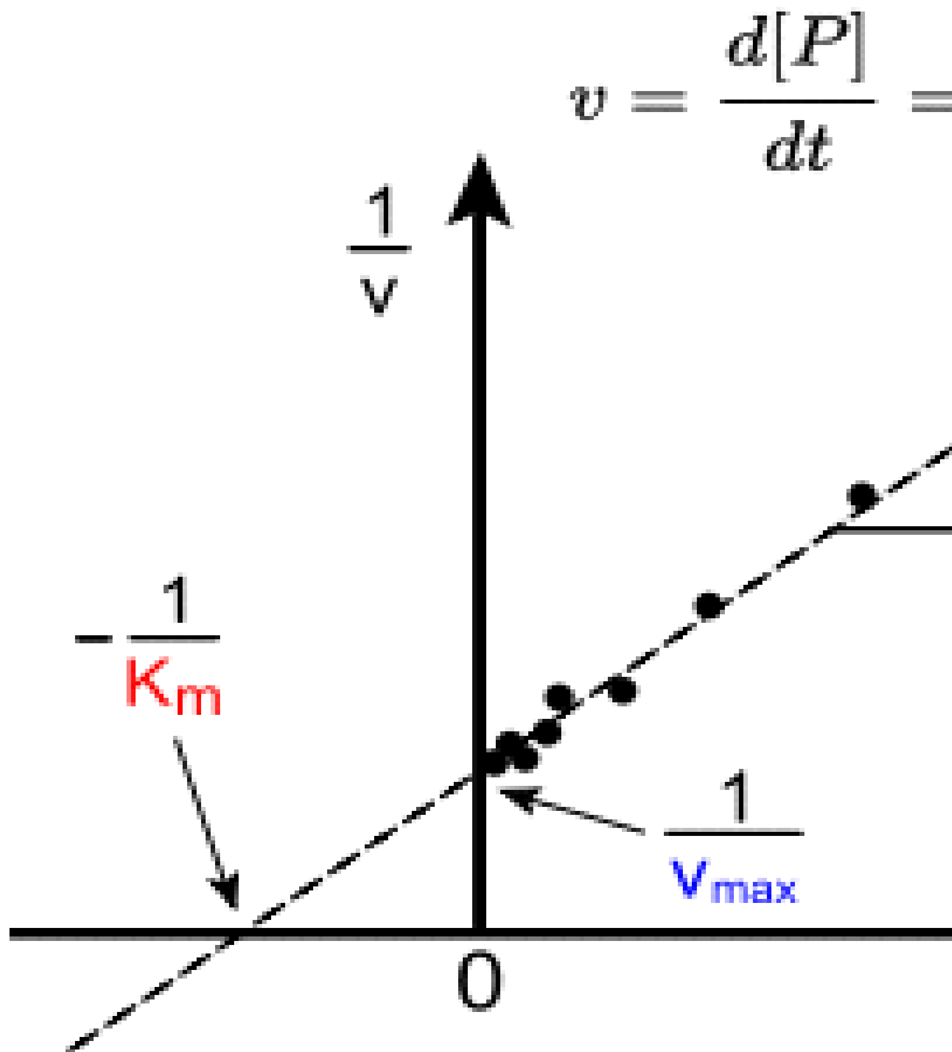

Figure 3: The Michaelis-Menten equation and determination of the kinetic parameters using a Lineweaver–Burk plot. (ʋ is the reaction rate; [S] is the concentration of substrate (TMB); V_{max} is the maximum rate achieved by the system, at saturating substrate concentration; the Michaelis constant K_M is the substrate concentration at which the reaction 27rate is half of V_{max}).

Note: The Michaelis-Menten equation can be rewritten as V= K_{Cat} [Enzyme] [S]/(K_M + [S]). K_{Cat} is measures the number of substrate molecules "turned over" by enzyme per second. The reciprocal of K_{Cat} is then the time required by an enzyme to "turn over" a substrate molecule. The higher the K_{Cat} is the more substrates get turned over in one second.

According to the data obtained from the Michaelis-Menten parameters, K_M and K_{Cat}, interpreted from the Lineweaver-Burk plots, 0.271 mM and 2265 s^{-1} were for the free HRP and 0.325 mM and 2158 s^{-1} for the rMAP/HA coacervate, containing HRP, respectively. In the opinion of the authors of study (Basak et al., 2005) similar Michaelis-Menten parameters observed for the free and encapsulated HRP indicate that the thin protective shell insignificantly affects the transport of the substrate to HRP, the enzymatic reaction kinetics, and the discharge of product. This type of behavior of encapsulated HRP is somehow similar to behaviors of HRP reported in the study of Basak et al. (2005) for the HRP encapsulated by nanogel crosslinked with N,N'-methylene bisacrylamide-based polymer. At the same time such a behavior is very different from behavior of HRP encapsulated in solid silica reported in the study of Sharma,(2005). These authors reported that due to encapsulation K_M was increased from 45.5 to 217.8 mM and K_{Cat} was decreased from $6.13 \cdot 10^7$ to $1.05 \cdot 10^5$ s^{-1} when o-dianisidine, whose molecular weight is similar to the TMB, was applied as the substrate.

We believe that the significantly enhanced thermal stability of the encapsulated HRP is due to the adsorption on the inner surface of the coacervate followed by covalent bonds with the coacervate, which prevent thermal fluctuations leading to enzyme deactivation at high temperature.

Conclusion

Thus, the study results indicate that the method of encapsulation of enzymes by using rMAP/HA coacervate-based procedure, proposed in the present paper is capable to provide significantly better results than other known encapsulation methods. The enzyme encapsulated in this way and the free enzyme show similar catalytic behavior, providing a robust enzyme model for a large variety of applications. Further investigation of this method hopefully will be able to extent the proposed approach to other important biological molecules.

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