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Immobilization of periodate-oxidized horseradish peroxidase by adsorption on sepiolite

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Abstract: Horseradish peroxidases (HRP), native and periodate-oxidized were immobilized onto sepiolite clay mineral by adsorption. Both peroxidases were adsorbed on this carrier in different quantities. Specific activity of immobilized enzymes was increased with increasing the amount of peroxidase added per gram of sepiolite. The highest specific activity was achieved when 15 mg of peroxidase was added per gram of sepiolite. Also, periodate-oxidized enzymes showed similar specific activity as native ones. Stability studies (pH, thermal and operational stability) were conducted for both peroxidases. Residual specific activity of HRP immobilized onto sepiolite declined with an increase of incubation time at 65 °C. Oxidized-peroxidase lost 64 % of the initial activity, whereas native HRP dropped 92 % of its activity after 5 minutes of incubation at 65 °C. Reduction of the enzyme activity was observed with the temperature increase from 30 to 80 °C. pH profiles of native peroxidase immobilized onto sepiolite were higher in both acidic and basic regions compared to periodate-oxidized enzyme. Oxidized HRP was more successful in studies of operational stability, it retained 42 % of its activity after 4 consecutive cycles of pyrogallol oxidation, whereas native peroxidase kept only 11 % of the original activity.

Keywords: modified enzyme; specific activity; thermostability; reusability.

INTRODUCTION

Enzymes, as biological catalysts, have shown a large potential for application in various fields of industry such as pharmaceutical, chemical, paper, food and cosmetic industries, as well as in the production of detergents and textile.[1,2] Immobilization of enzymes on different support materials allows for a higher

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stability of enzyme, reduces an enzyme inactivation and prevents a product contamination.[3,4] Additionally, reusability and higher cost-effectiveness are accomplished by enzyme immobilization. It is important to stress that this method also allows for easy separation of the immobilized enzyme from the reaction mixture and, thus, its recovery. Different types of enzyme immobilization methods, such as adsorption, covalent binding, cross linking and entrapment, can be used.[5] Each of these methods has its own benefits and drawbacks. Adsorption implies binding between an enzyme and support via mostly weak bonds (van der Waals, electrostatic interactions and hydrogen bonds). This is a simple and cost-effective method, however a leakage and inactivation of enzyme occur as common side effects. Covalent binding of an enzyme to a carrier provides strong linkage, thus preventing highly undesirable enzyme leakage.[5,6] Entrapment of an enzyme is a physical method which does not involve formation of covalent bonds between an enzyme and support, thereby increases the possibility of enzyme leakage.[7]

In order to be suitable in enzyme immobilization reactions criteria regardless of the enzyme immobilization type, carriers need to fulfil certain criteria, such as high surface area, high permeability and hydrophilicity, microbial, mechanical, chemical and thermal stability. Based on their chemical properties, carriers can be classified into two major groups: inorganic and organic.[8] First supports used for the enzyme immobilization were inorganic owning to their high mechanical strength, thermal stability and resistance to organic solvents. They are suitable for application in different fields of industry, whereas organic carriers, due to their high reactivity, have found greater application in laboratory conditions.

As a consequence of great abundance of naturally present clay minerals, their role in many fields of industry, as well as in the scientific research, increases.[9] Among them, the most commonly used are sepiolite, palygorskite, montmorillonite and kaolinite. Their availability, physicochemical properties and crystal structure allow for their potential applications in many different fields. These materials are economic and can be used without any additional modifications. Active groups presented on the surface of these materials (carboxyl, hydroxyl, thiol, amine groups) make them suitable for binding of many different enzymes in immobilization reactions.[9] Sepiolite ($Si_{12}O_{30}Mg_8(OH)_4(OH_2)_4 \times nH_2O$), as a silicate clay mineral rich with magnesium, is characterized by a fibrous crystal structure, great specific surface area and active silanol groups on the external surface.[10] Each of these properties makes sepiolite an excellent candidate for adsorption reactions in many different areas. The world's sepiolite reserves are estimated to be about 8 million tons and the mine with the largest amount of sepiolite is located in Spain. Sepiolite often appears in two forms, α and β-sepiolite, differing in the crystal shape. α-sepiolite consists of a large bundle of fibrous crystals, whereas β-sepiolite comprises short and thin fibrous crystals. α-Sepiolite is abundant in Tertiary rocks rich in phosphates, salts, zeolites, while β-sepiolite

prevails in marine, lagoonal and pedogenic environments. Change in the nature and localization of functional groups on the surface of sepiolite alters mechanical properties of the whole molecule and therefore expands the range of areas in which it can be applied.[10]

Sepiolite has already been used as a carrier for various enzymes. Olshansky and co-workers immobilized by adsorption laccase derived from *Rhus vernicifera* on sepiolite and modified sepiolite (with Cu(II) and chitosan). The immobilized enzyme on unmodified Cu-chitosan- and chitosan-modified sepiolite has shown increased activity of 250, 700, and 500 % compared to the free form.[11] Alkaline phosphatase from the bovine intestinal mucous membrane was immobilized by sorption on Na-sepiolite. In a wide range of pH (from 5 to 11), the immobilized enzyme has shown substantially higher activity than the soluble enzyme. The authors have reported lower stability when storing at 30 °C and higher thermal denaturation of the immobilized alkaline phosphatase compared to its free form.[12] Sedaghat and co-workers immobilized alkaline phosphatase from calf intestinal mucous membrane on unmodified and modified sepiolite (with mono- and bilayer surfactant). The enzyme immobilized on sepiolite with the bilayer surfactant coverage has shown the most promising temperature and pH stability. Also, this immobilization system has shown the same activity as the free enzyme, whereas the enzyme immobilized on unmodified sepiolite and modified sepiolite with monolayer surfactant coverage has shown reduced activity by about 30 and 38 %.[13] Shirvani and coworkers have studied the adsorption of alkaline phosphatase on polygorskite and sepiolite. They found the enzyme activity loss after immobilization on these two carriers. However, higher resistance to a Cd inhibitory effect was achieved with immobilization.[14] Sepiolite and bentonite have been used as carriers for catalase, and an effect of immobilization on thermal, operational, and storage stability has been studied. Compared to the free enzyme, immobilized catalase has shown improved properties, with the most promising thermal, operational, and storage stability achieved by immobilization on sepiolite.[15] Mortazavi and coworkers immobilized α-amylase from *Bacillus subtilis* and lipase from *Candida rugosa* via adsorption on modified Na-sepiolite. Lipase immobilized on modified sepiolite with hydrophobic properties and α-amylase immobilized on modified sepiolite with hydrophilic properties have shown improved thermal and storage stability, as well as reusability when compared to free enzyme.[16]

Peroxidases are enzymes that are capable of oxidizing phenolic compounds, the most frequent water pollutants, in the presence of hydrogen peroxide. This oxidation reaction results in the formation of water-insoluble polymeric aggregates, effortlessly eliminated from the aqueous phase by filtration or sedimentation. Among all enzymes from the group of peroxidases, horseradish peroxidase (HRP) has been the most commonly applied for this purpose.[17,18] A

main issue in using enzymes for water treatment is their inactivation, most probably caused by interactions between phenoxy radicals and the enzyme's active site.[19] This problem can be overcome by the enzyme immobilization.

In the present work, β-sepiolite clay mineral, obtained from a site near Obrenovac, was used for the first time as a carrier for immobilizing horseradish peroxidase (HRP, EC 1.11.7). Also, native HRP was, for the first time, immobilized by adsorption after periodate oxidation of the carbohydrate part of the protein molecule onto sepiolite. Activity, thermal, and operational stability were tested to study the effect of enzyme modification by periodate oxidation on the activity and stability of sepiolite-adsorbed HRP.

EXPERIMENTAL

Materials

HRP (150–250 U mg^{-1}), pyrogallol used as a substrate for the peroxidase oxidation reaction and sodium periodate were purchased from Sigma-Aldrich (USA). Hydrogen peroxide was obtained from AppliChem GmbH (Darmstadt, Germany). Sodium dihydrogen phosphate anhydrous and sodium acetate were purchased from Centrohem (Stara Pazova, Serbia) and Fluka (Buchs, Switzerland), respectively. Glycerol (from plant, for laboratory use) was purchased from Serva (Heidelberg, Germany).

Sepiolite

Sepiolite samples were collected near Obrenovac (Serbia) and milled with a pestle and mortar to the fineness of particles of 0.3 mm. Milled sepiolite was subsequently rinsed with distilled water and smaller particles were removed by fractional sedimentation. Procedure was repeated several times.

Oxidation of HRP

Oxidation of HRP was performed with the 5 mmol L^{-1} sodium periodate solution in sodium acetate buffer pH 5.0 (50 mmol L^{-1}) in the dark at 4 ºC for 6 h. By adding glycerol to a final concentration of 0.2 % (v/v), the oxidation reaction was stopped. Oxidized HRP was dialyzed overnight against sodium acetate buffer pH 5.

Immobilization of HRP onto sepiolite clay mineral

Sepiolite (0.1 g) was rinsed with 5 mL of sodium acetate buffer pH 5.0 (50 mmol L^{-1}) and incubated for 48 h with different amounts of native or oxidized HRP (1, 5, 15 and 25 mg g^{-1}) per gram of clay mineral. Sepiolite with immobilized enzyme was subsequently rinsed with 2 mL of sodium acetate buffer pH 5.0 (0.1 mol L^{-1}) and stored in the same buffer at 4 ºC until further use.

Washings were collected and used for determination of the unbound enzyme activity.

Activity studies of immobilized enzyme

Pyrogallol and hydrogen peroxide (H_2O_2) were used as substrates in an assay used to determine the peroxidase activity. Ten μL of enzyme dilution from the washings and 10 μL of H_2O_2 (9.7 mmol L^{-1}) were introduced into 1 mL of the pyrogallol solution (13 mmol L^{-1}) in sodium phosphate buffer pH 7 at room temperature. Absorbance was measured at 420 nm for 3 minutes using UV-VIS spectrophotometer (Shimadzu Corporation UV-2501PC, Japan). Value of the enzyme activity was calculated from the absorbance coefficient of purpurogallin (12 mg^{-1} cm^{-1}). The activity of immobilized enzyme was determined by introducing 9.0 mg of sepiolite

with immobilized HRP and 30 μL of H_2O_2 into 3 mL of pyrogallol. Every 60 s aliquots were sampled from the mixture, filtrated and the absorbance at 420 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that produces 1 mg of purpurogallin in 20 s at 20 ºC. The specific activity of enzyme was calculated per gram of dry weight of sepiolite. All measurements were done in triplicate.

Temperature stability kinetics of immobilized HRP

Temperature stability of native and periodate oxidized HRP and afterward immobilized onto sepiolite clay mineral (15 mg/g) was monitored at 65 °C for 5 and 30 minutes. An appropriate amount of the enzyme immobilized onto sepiolite was incubated in sodium phosphate buffer pH 7.0 (0.1 mol L^{-1}) at 65 °C a certain period of time. The immobilized enzyme was subsequently cooled down to room temperature. Residual specific activity of the immobilized enzyme was determined as described previously.

Activity measurements at different pH values

To monitor the enzymatic activity of immobilized HRP, a series of 0.1 mol L^{-1} phosphate-citrate buffers with pH values from 2.0 to 8.0 was used. In order to determine the enzyme stability at pH 9.0, sodium-glycinate buffer (0.1 mol L^{-1}) was applied. According to the above-described procedure, relative activity of HRP immobilized onto sepiolite clay mineral was examined. Obtained results were afterwards normalized to the maximum activity at optimum pH, i.e. the relative activity, expressed as a percentage and presented.

Determination of thermostability of soluble and immobilized HRP

Thermostability of immobilized HRP at different temperatures (30, 40, 50, 60, 70 and 80 °C) was examined by incubating for 30 min of the enzyme immobilized on sepiolite in sodium phosphate buffer pH 7.0 (0.1 mol L^{-1}) at given temperatures. Subsequently, the immobilized enzyme was cooled down to room temperature and the relative activity was measured according to the procedure described above.

Reusability studies

Operational stability of immobilized peroxidase was determined by conducting pyrogallol oxidation in a batch reactor for several consecutive cycles at room temperature (25 ºC). At the end of each cycle, sepiolite with the immobilized enzyme (native and oxidized) was rinsed couple of times with sodium phosphate buffer pH 7.0 (0.1 mol L^{-1}). This procedure was repeated several times with fresh aliquots of both substrates, pyrogallol and H_2O_2.

RESULTS AND DISCUSSION

Immobilization of an enzyme on different support materials provides the improved enzyme activity and stability at elevated temperatures, as well as satisfactory stability in organic solvents and at different pH values.[20] All of the above-mentioned facts allow for better reusability and longer storage stability, which is of great importance for potential applications. When naturally present clay minerals such as sepiolite or palygorskite are used for the enzyme immobilization, satisfactory results in terms of the enzyme activity and stability have been obtained. Shirvani and co-workers have studied adsorption of alkaline phosphatase on polygorskite and sepiolite. They found a loss of enzyme activity after immobilization on these two carriers. The enzyme lost 7.5-23.1 % and 9.8-28.8 % of its activity by immobilizing on polygorskite and sepiolite, depending on

the substrate concentration. However, the immobilized enzymes have been substantially more resistant to the inhibitory effect of Cd than the free enzyme.[14] Laccase derived from *Rhus vernicifera* immobilized by adsorption on sepiolite has shown an increased activity by 250 %.[11] Catalase immobilized on sepiolite has shown improved thermal, storage, and operational stability in comparison to the free enzyme.[15]

In our research sepiolite was used as a carrier for the adsorption of HRP (native and periodate-oxidized) (Figure 1). Subsequently, the effect of immobilization on the stability (thermal, pH, etc.) of both native and oxidized enzymes was examined.

Fig. 1 Sepiolite clay mineral. The size of the particles was 50±20μm.

In order to perceive a difference in activity and stability of native and periodate-oxidized HRP, both immobilized onto sepiolite, obtained results are compared (Figure 2). A formerly optimized method for hydrolases and invertase – the periodate method, involves binding to a support material through enzyme carbohydrate moiety previously subjected to oxidation with sodium periodate.[21-24]

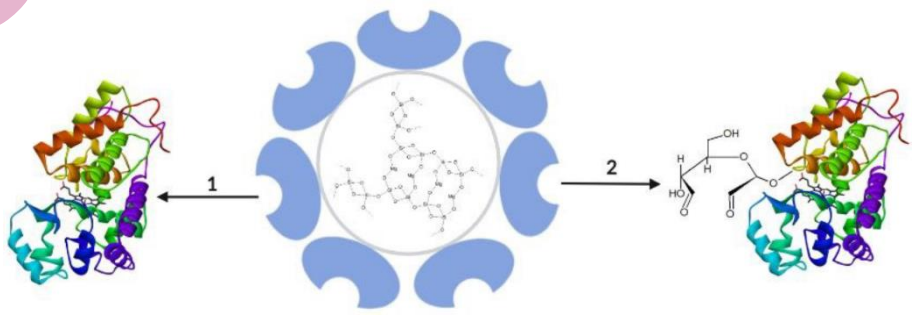

Fig. 2 Adsorption of HRP on sepiolite: (1) native and (2) oxidized enzyme

Oxidation with sodium periodate creates aldehyde groups (Figure 2) that could react with amino groups within the enzyme molecule leading to cross-linking and increase in adsorption efficiency and enzyme stability.

Different amounts of both native and periodate-oxidized enzyme are added per gram of sepiolite (25, 15, 5 and 1 mg g^{-1}) and specific activities of immobilized enzymes are calculated. When concerning native HRP immobilized onto sepiolite clay mineral, an increase in the amount of enzyme added per gram of the support results in enhanced specific activity of the immobilized enzyme (Figure 3). Further growth in the amount of added enzyme leads to a decrease in the specific activity. The maximum enzyme specific activity is reached when the amount of 15 mg of peroxidase is immobilized per gram of sepiolite. Different results are obtained when oxidized HRP was immobilized onto sepiolite. Specific activity increases with increasing the amount of enzyme added per gram of sepiolite. Compared to the oxidized enzyme, similar specific activities of native HRP are detected.

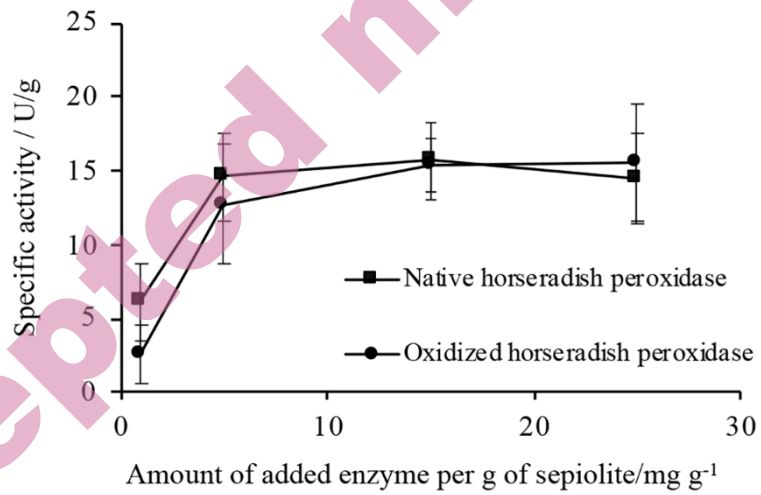

Fig. 3 Effect of the amount of added enzyme on the specific activity of immobilized HRP (native and oxidized). Standard error was calculated from triplicate measurements of the specific activity.

The obtained results for native and periodate-oxidized enzymes show that at 25 mg of the enzyme loading per gram of sepiolite native peroxidase has the specific activity of 14.4 U g^{-1}, whereas oxidized HRP show 15.5 U g^{-1}. The trend is slightly different for other concentrations of the enzyme thus no general conclusions can be drawn. These findings are corroborated with results obtained by Öztürk *et al.* which have shown that excessive loadings of lipase onto sepiolite and montmorillonite clay minerals resulted in lower immobilization efficiency.[25] They have found that the optimal lipase content was 7.5 mg per gram of both clay

minerals. Higher amounts of the immobilized enzyme can lead to loss of the enzyme activity due to smaller accessibility of the enzyme to the substrate. Our study shows a rise of the specific activity with increasing the peroxidase loading onto sepiolite clay mineral up to 15 mg of enzyme per gram of sepiolite for native peroxidase. Further increase in the enzyme loading does not lead to a significant change of the specific activity, which may be a result of burring of the active site with the excess of the enzyme, a steric hindrance on the access of the substrate, disarrangement of the three-dimensional structure of the enzyme or diffusional limitations.[13] With the increase in the amount of immobilized peroxidase, the specific activity of the periodate-oxidized enzyme raises, reaches a plateau and remains unaltered.

Thermostability

In order to examine how immobilization and periodate oxidation affect the thermostability of sepiolite adsorbed HRP, the enzymes immobilized in the amount of 15 mg per g of sepiolite was first incubated at 65 °C for different time periods (Figure 4). Values of residual activities of native and periodate-oxidized HRPs show that more stable biocatalyst is the one previously oxidized with periodate. Periodate-oxidized enzyme preserves 36 % of residual activity after 5 min of incubation, whereas the native HRP retains only 8 % of residual activity. By increasing incubation time at 65 °C to 30 minutes the activity of both HRPs immobilized onto sepiolite substantially decreases to 6 % for periodate oxidized and 4 % for native HRP.

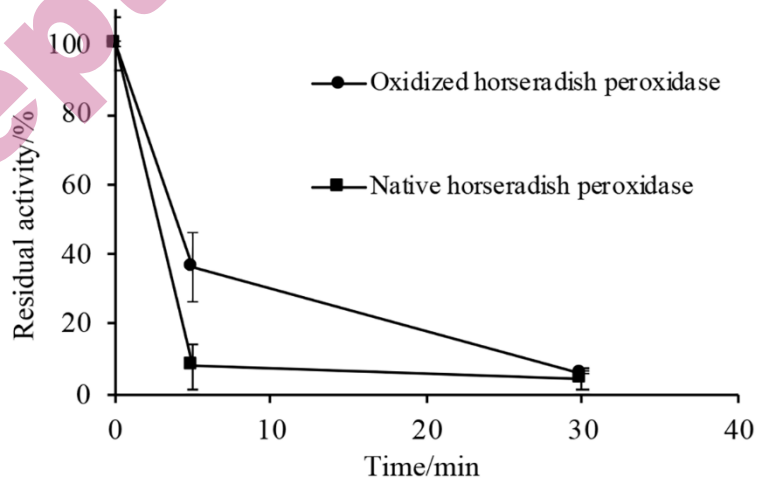

Fig. 4 Effect of incubation time at 65 °C on residual activities of native and periodate-oxidized HRPs immobilized onto sepiolite clay mineral by adding 15 mg of the enzyme per g of sepiolite.

Native peroxidase lost around 92 % of its initial activity after incubation at 65 °C for 5 minutes, whereas peroxidase oxidized with sodium periodate and immobilized onto sepiolite lost 64 % of the initial activity. Thus, periodate-oxidized HRP is more stable at elevated temperatures than native peroxidase immobilized onto sepiolite. Similar results have been obtained for catalase immobilized onto sepiolite and bentonite.[15] Sepiolite immobilized catalase retained 19.1 % of its initial activity at 65 °C after 1 h of incubation.

In further experiments for thermostability measurements, we have used a 30-minute incubation period at different temperatures for different amounts of adsorbed enzyme.

The obtained results show the identical temperature stability for all used concentrations of immobilized enzymes at 40 °C. Data presented in Figure 5 show dependence of incubation at different temperatures on the residual activity of oxidized HRP immobilized onto sepiolite, whereas results obtained for immobilized native peroxidase are not shown. The increase in the temperature from 40 to 80 °C, leads to the gradual decrease of the enzyme residual activity. At 60 °C periodate-oxidized HRP retains almost 30 % of the initial activity. Further raise of temperature results in the decline of the activity by almost 20 %. These findings are corroborated with those reported for immobilized invertase.[26] Immobilized invertase preserved almost 85 % of its activity during incubation at 50 °C, whereas free invertase lost 50 % of the initial activity under the same conditions. After incubation at 70 °C, the free enzyme lost all activity, however immobilized invertase retained 80 % of its activity. Immobilization of HRP on perlite led to higher thermostability at 80 °C.[27] The immobilized enzyme retained 30 % of the initial activity after incubation at 80 °C for 20 minutes. Kim and co-workers immobilized HRP on fulvic acid-activated montmorillonite K-10. They compared the thermal stability of the immobilized and free enzyme and found the markedly higher relative activity of immobilized HRP over the relatively high-temperature range (75 % and around 55 % for immobilized and free HRP, respectively at 45 °C.[28] Zhang *et al.* found the activity loss of free HRP of around 18 % caused by increasing temperature from 20 to 60 °C, whereas the activity of hemin-histamine-montmorillonite conjugates increased by 3.6 times in the same temperature range.[29] The complete loss of the free enzyme activity at 70 °C as a consequence of protein denaturation has been reported in the literature.[30,31] Lower stability at higher temperatures may be due to loss of the heme group during incubation. Chattopadhyay *et al.* reported a drastic change in the overall secondary structure of the enzyme at 74 °C, associated with the complete release of the heme moiety from the enzyme.[32]

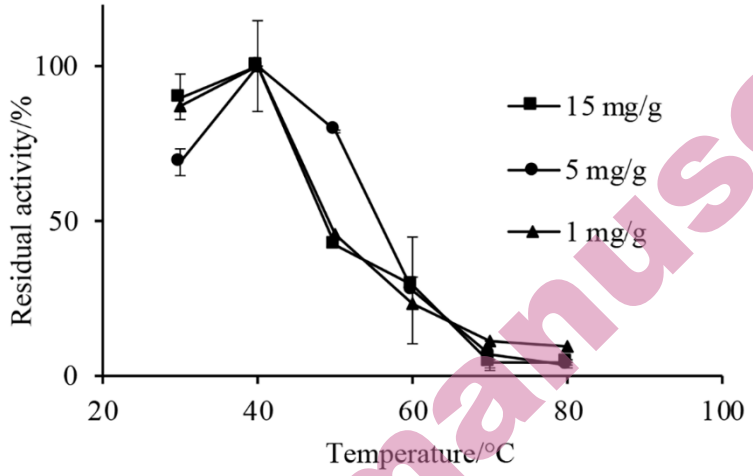

Fig. 5 Effect of incubation at different temperatures on the residual activity of periodate-oxidized HRP immobilized on sepiolite

pH optimum

The change of pH values was monitored in the range from 2.0 to 9.0 for both native and HRP oxidized with sodium periodate, and immobilized on the same carrier using 15 mg of the enzymes per gram of the sepiolite carrier (Figure 6).

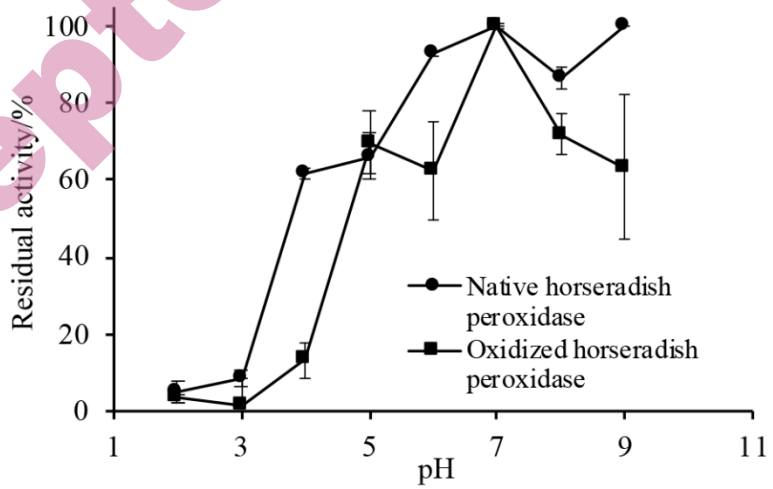

Fig. 6 Effect of pH on activities of native and oxidized HRP immobilized on sepiolite

Both peroxidases show the same trend in terms of relative activities. The increase in pH values from acidic to basic leads to a gradual raise of relative activities until reaching the pH optimum (pH 7.0). Subsequently, the relative

activities decline. The optimum pH values for both peroxidases (native and periodate-oxidized) overlap. The bell-shaped pH profiles demonstrate higher stabilities of sepiolite immobilized native peroxidase than periodate-oxidized HRP immobilized onto sepiolite in both acidic and basic regions. Optimum pH obtained in our study was corroborated with the study of Torabi *et al.*[23] Peroxidase immobilized onto perlite showed optimum at pH 7.0 and a wider pH range for native enzyme compared to immobilized peroxidase.[27] This effect is also observed in our study and can be explained by different behaviour of native enzyme in slightly acidic regions. The maximum activity of HRP immobilized on kaolin is at pH 5.0. As suggested by the authors, the enzyme binding at this pH allowed for the conservation of the catalytic function.[33] Liu *et al.* have shown that HRP immobilized on silane-modified ceramics showed higher acid-base stability than free HRP. Immobilized enzymes preserved their activity in a wide range of pH due to the reduced effect of pH alternation in the enzyme vicinity caused by the conditioning of the microenvironment. The maximum activity of immobilized enzyme was at pH 6 whereas that of free HRP was at pH 7.[34] Kim and co-workers have reported the optimal pH of free HRP and montmorillonite immobilized HRP of 8 and 9, respectively. This shift of the pH optimum to more alkaline conditions has been related to unequal H+ displacement in the microenvironment around the immobilized enzyme, change in the microenvironment around the immobilized HRP towards a cationic environment, and separation of the enzyme from bulk as well as to the charge of clay. The immobilized enzyme has shown better activity than the free enzyme over a wide range of pH, which is attributed to the protection effect achieved by the stable binding to the inorganic carrier that restricts denaturation or unfolding of the enzyme due to sudden pH changes.[28] Sedaghat *et al.* have reported the dame pH optimum of free HRP, and HRP immobilized on sepiolite, sepiolite with bilayer, and monolayer surfactant coverage (pH 10) with the broader profile of the HRP immobilized on sepiolite with bilayer surfactant coverage.[13]

Operational stability and enzyme leakage

The same batch of immobilized enzymes (native and periodate-oxidized) is used in several consecutive cycles for pyrogallol oxidation to examine the operational stability. Residual activities of the immobilized enzymes are monitored for 4 cycles, with each cycle lasting 180 minutes. This time period was chosen based on our previous study showing that longer cycles provide higher amounts of solid oxidation products that block the pores of the carrier and, thus, the immobilized enzyme inside the pores, disabling its further catalytic activity.[35] Between two cycles sepiolites with immobilized HRP are rinsed several times with sodium phosphate buffer and subsequently used for another round of pyrogallol oxidation. The enzyme activity obtained in the first cycle is considered 100 %. When comparing values of residual activities for both native and oxidized HRP,

especially at the concentration of 1 mg g^{-1} that we have shown in order to detect sooner leakage of the enzyme and loss of the activity, higher operational stability is achieved with the periodate-oxidized enzyme (Figure 7). Native HRP retains only 11 % of its initial activity after 4 consecutive cycles of pyrogallol oxidation. HRP oxidized with sodium periodate and immobilized onto sepiolite shows better results in terms of the residual activity – after 4 cycles of pyrogallol oxidation it preserves 42 % of the initial activity. The operational stability of aldehyde dehydrogenase (ALDH) immobilized onto montmorillonite was monitored for several cycles.[24] The activity of the immobilized enzyme decreased by 20 % after each cycle, thus it dropped to barely 24 % by the end of the fifth cycle.

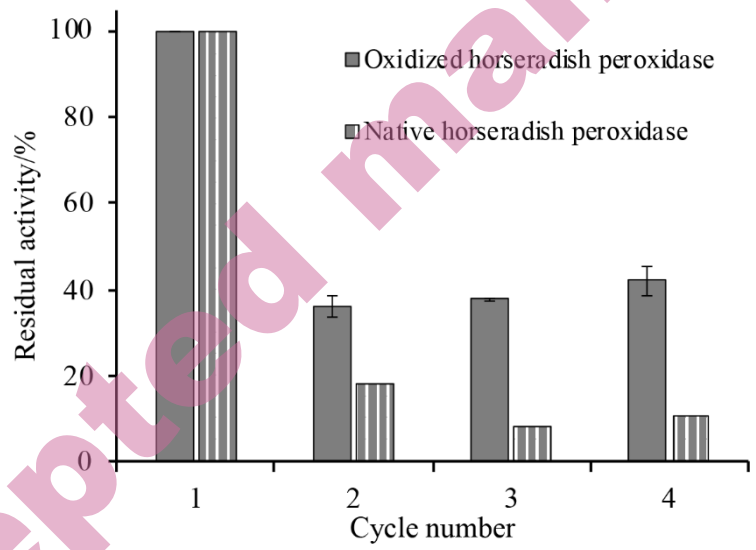

Fig. 7 Catalytic activity of native and oxidized HRP during repeated use

Residual activities of different amounts of periodate-oxidized peroxidases immobilized per gram of sepiolite are shown on Figure 8. After 4 cycles of repeated use in pyrogallol oxidation, the enzyme immobilized in the amount of 1 mg per gram of sepiolite shows the most promising results regarding the residual activity. It retains 42 % of the initial activity, whereas the enzyme immobilized in other concentrations shows slightly different results. Generally, the same trend is observed for all enzyme concentrations. An increase in the number of cycles leads to a decline of residual activities. Reduction of the specific activity after each cycle for lipase and α-amylase immobilized onto sepiolite has been reported in literature.[36] It has been addressed to either accumulation of water onto the sepiolite surface or enzyme inactivation. Inactivation of the enzyme, which results from blocking of the active site of the enzyme by radical products formed in oxidation reactions or the enzyme leakage from the carrier surface, can cause the activity

loss during the repetitive use. In our study the periodate oxidation of HRP forms aldehyde groups and allows for cross-linking of HRP protein molecules during adsorption, thus decreasing the possibility of leakage and increasing the stability.

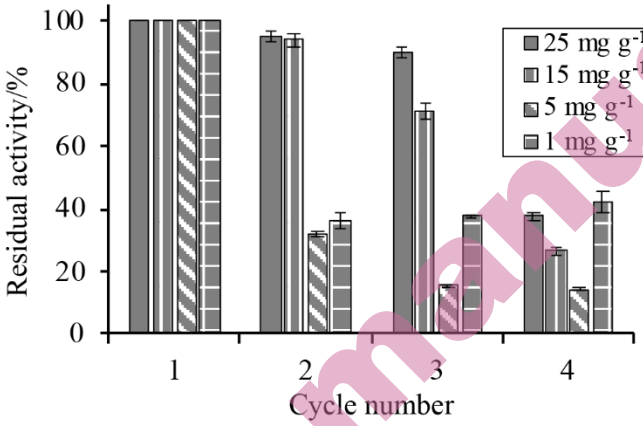

Fig. 8 Catalytic activity of periodate-oxidized HRP immobilized on sepiolite (different concentrations of the enzyme)

CONCLUSION

Sepiolite clay mineral was used for the immobilization of HRP. In order to determine whether difference in the binding affinity and stability of native and periodate-oxidized HRPs exists, several studies were performed with this enzyme for the first time. Effect of the amount of enzyme added per gram of sepiolite on the specific activity was examined. It was found that the specific activity raised with increasing the quantity of added enzyme. Fifteen mg of peroxidase per gram of the carrier provided the maximum specific activity. Similar values of the specific activity were obtained for oxidized peroxidases in comparison to the native enzyme. Both native and oxidized HRP immobilized onto sepiolite were tested in various stability studies (pH, thermal and operational stability). Incubation of enzymes at 65 °C for an appropriate period of time showed increased stability at elevated temperatures for periodate oxidized HRP. Activity of immobilized peroxidases was also examined at various temperatures (30, 40, 50, 60, 70 and 80 °C). Obtained results showed gradual decrease of the enzyme stability and activity with increasing temperature from 30 to 80 °C. The bell-shaped pH profiles were observed for both peroxidase (native and oxidized) immobilized onto sepiolite clay mineral. Operational stability was examined by using the same batch of immobilized enzymes for pyrogallol oxidation. Native HRP retained only 11 % of the initial activity after 4 consecutive cycles, whereas the oxidized enzyme preserved 42 % of the activity after the same number of cycles. All results indicate that periodate oxidation of HRP prior to adsorption on

carriers such as sepiolite provided more stable immobilized enzyme probably as a result of formation of aldehyde groups that can cross-link enzyme molecules via amino groups and additionally stabilize adsorbed HRP. This method can generally apply for other catalytic glycoproteins.[22]

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И З В О Д

ИМОБИЛИЗАЦИЈА ПЕРЈОДАТНО ОКСИДОВАНЕ ПЕРОКСИДАЗЕ ИЗ РЕНА АДСОРПЦИЈОМ НА СЕПИОЛИТУ

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Пероксидазе из рена (ХРП), нативна и оксидована перјодатом, су имобилизоване адсорпцијом на минерал глине сепиолита. Обе пероксидазе су адсорбоване на овом носачу у различитим количинама. Специфична активност имобилисаних ензима се повећава са повећањем количине додате пероксидазе по граму сепиолита. Највећа специфична активност је постигнута додавањем 15 mg пероксидазе по граму сепиолита. Поред тога установљено је и да перјодатно оксидовани ензими имају сличну специфичну активност као и нативни. За обе пероксидазе је одређена pH, термичка и оперативна стабилност. Установљено је да преостала специфична активност ХРП имобилисане на сепиолит опада са повећањем времена инкубације на 65 °C. Оксидована пероксидаза је изгубила 64 % почетне активности, док је код нативне ХРП активности опала за 92 % након 5 минута инкубације на 65 °C. Са повећањем температуре од 30 до 80 °C смањује се активност ензима. И у киселом и у базном региону pH профил нативне пероксидазе имобилисане на сепиолиту је виши у поређењу са pH профилом перјодатно оксидованог ензима. Установљено је да је оперативна стабилност оксидоване ХРП знатно боља него оперативна стабилност нативне пероксидазе. Перјодатно оксидована пероксидаза је задржала 42 % своје активности након 4 узастопна циклуса оксидације пирогалола, док је нативна задржала само 11 % првобитне активности.

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REFERENCES

1. C. L. B. Reis, E. Y. A. de Sousa, J. de F. Serpa, R. C. Oliveira, J. C. S. dos Santos, *Quim. Nova* **42** (2019) 768. (https://doi.org/10.21577/0100-4042.20170381)
2. S. Cantone, V. Ferrario, L. Corici, C. Ebert, D. Fattor, P. Spizzo, L. Gardossi, *Chem. Soc. Rev.* **42** (2013) 6262 (https://doi.org/10.1039/C3CS35464D)

3. D. M. Liu, J. Chen, Y. P. Shi, *TrAC - Trends Anal. Chem.* **102** (2018) 332 (https://doi.org/10.1016/j.trac.2018.03.011)
4. C. Garcia-Galan, Á. Berenguer-Murcia, R. Fernandez-Lafuente, R. C. Rodrigues, *Adv. Synth. Catal.* **353** (2011) 2885 (https://doi.org/10.1002/adsc.201100534)
5. A. A. Homaei, R. Sariri, F. Vianello, R. Stevanato, *J. Chem. Biol.* **6** (2013) 185 (http://dx.doi.org/10.1007/s12154-013-0102-9)
6. B. Brena, P. González-Pombo, F. Batista-Viera, *Immobilization of Enzymes and Cells: Third Edition*, *Methods in Molecular Biology* **1051** (2013) 15 (https://doi.org/10.1007/978-1-62703-550-7_2)
7. Z. Ashkan, R. Hemmati, A. Homaei, A. Dinari, M. Jamlidoost, A. Tashakor, *Int. J. Biol. Macromol.* **168** (2020) 708 (https://doi.org/10.1016/j.ijbiomac.2020.11.127)
8. A. Homaei, *Enzyme immobilization and its application in the food industry*, in *Advances in Food Biotechnology*, R. V. Rai, John Wiley & Sons, Hoboken, New Jersey, USA, 2015, p. 145 (https://doi.org/10.1002/9781118864463.ch09)
9. A. R. Ismail, K. H. Baek, *Int J Biol Macromol* **163** (2020) 1624 (https://doi.org/10.1016/j.ijbiomac.2020.09.021)
10. F. Largo, R. Haounati, S. Akhouairi, H. Ouachtak, R. El Haouti, A. El Guerdaoui, N. Hafid, Diogo M. F. Santos, F. Akbal, A. Kuleyin, A Jada, A. A. Addi, *J. Mol. Liq.* **318** (2020) 114247 (https://doi.org/10.1016/j.molliq.2020.114247)
11. Y. Olshansky, S. Masaphy, R. A. Root, G. Rytwo, *Appl. Clay Sci.* **152** (2018) 143 (https://doi.org/10.1016/j.clay.2017.11.006)
12. M.S. Carrasco, J.C. Rad, S. Gonzalez-Carcedo, *Biores. Technol.* **51** (1995) 175 (https://doi.org/10.1016/0960-8524(94)00115-H)
13. M. E. Sedaghat, M. Ghiaci, H. Aghaei, S. Soleimanian-Zad, *Appl. Clay Sci.* **46** (2009) 131 (https://doi.org/10.1016/j.clay.2009.07.021)
14. M. Shirvani, B. Khalili, M. Kalbasi, H. Shariatmadari, F. Nourbakhsh, *Clays Clay Miner.* **68** (2020) 287 (https://doi.org/10.1007/s42860-020-00066-w)
15. S. Cengiz, L. Çavaş, K. Yurdakoç, *Appl. Clay. Sci.* **65–66** (2012) 114 (https://doi.org/10.1016/j.clay.2012.06.004)
16. S. Mortazavi, H. Aghaei, *Int. J. Biol. Macromol.* **164** (2020) 1 (https://doi.org/10.1016/j.ijbiomac.2020.07.103)
17. N. Caza, J. K. Bewtra, N. Biswas, K. E. Taylor, *Water Res.* **33** (1999) 3012 (https://doi.org/10.1016/S0043-1354(98)00525-9)
18. F. Quintanilla-Guerrero, M. A. Duarte-Vázquez, B. E. García-Almendarez, R. Tinoco, R. Vazquez-Duhalt, C. Regalado, *Biores. Technol.* **99** (2008) 8605 (https://doi.org/10.1016/j.biortech.2008.04.031)
19. I. Alemzadeh, S. Nejati, *J. Haz. Mat.* **166** (2009) 1082 (https://doi.org/10.1016/j.jhazmat.2008.12.026)
20. N. Miletić, A. Nastasović, K. Loos, *Biores. Technol.* **115** (2012) 126 (https://doi.org/10.1016/j.biortech.2011.11.054)
21. Z. Knezevic, N. Milosavic, D. Bezbradica, *Biochem. Eng. J.* **30** (2006) 269 (https://doi.org/10.1016/j.bej.2006.05.009)
22. R. M. Prodanović, M. B. Simić, Z. M. Vujčić, *J. Serb. Chem. Soc.* **68** (2003) 819 (https://doi.org/10.2298/JSC0311819P)
23. N. Milosavić, R. Prodanović, S. Jovanović, Z. Vujčić, *Enzyme Microb. Technol.* **40** (2007) 1422 (https://doi.org/10.1016/j.enzmictec.2006.10.018)
24. R. Prodanović, S. Jovanović, Z. Vujčić, *Biotechnol. Lett.* **23** (2001) 1171 (https://doi.org/10.1023/A:1010560911400)

25. H. Öztürk, E. Pollet, V. Phalip, Y. Güvenilir, L. Avérous, *Polymers* **8** (2016) 416 (https://doi.org/10.3390/polym8120416)
26. H. N. Oztop, C. Hepokur, D. Saraydin, *J. Food Sci.* **74** (2009) N45 (https://doi.org/10.1111/j.1750-3841.2009.01302.x)
27. S. F. Torabi, K. Khajeh, S. Ghasempur, N. Ghaemi, S. O. R. Siadat, *J. Biotechnol.* **131** (2007) 111 (https://doi.org/10.1016/j.jbiotec.2007.04.015)
28. H. J. Kim, Y. Suma, S. H. Lee, J. A. Kim, H. S. Kim, *J. Mol. Catal. B Enzym.* **83** (2012) 8 (https://doi.org/10.1016/j.molcatb.2012.06.012)
29. L. Zhang, C. Gu, J. Xiong, M. Yang, Y. Guo, *Sci. China Chem.* **58** (2015) 731 (https://doi.org/10.1007/s11426-014-5196-6)
30. M. Kurosawa, T. Itoh, Y. Kodera, A. Matsushima, M. Hiroto, H. Nishimura, Y. Inada, *Bioconjugate Chem.* **13** (2002) 167 (https://doi.org/10.1021/bc000133+)
31. J. Xiong, C. Hang, J. Gao, Y. Guo, C. Gu, *Chem. Eng. J.* **254** (2014) 276 (https://doi.org/10.1016/j.cej.2014.05.139)
32. K. Chattopadhyay, S. Mazumdar, *Biochemistry* **39** (2000) 263 (https://doi.org/10.1021/bi990729o)
33. N. Ž. Šekuljica, N. Ž. Prlainović, J. R. Jovanović, A. B. Stefanović, V. R. Djokić, D. Ž. Mijin, Z. D. Knežević-Jugović, *Bioprocess Biosyst. Eng.* **39** (2016) 461 (https://doi.org/10.1007/s00449-015-1529-x)
34. W. Liu, W. C. Wang, H. S. Li, X. Zhou, *Water Sci. Tech.* **63** (2011) 1621 (https://doi.org/10.2166/wst.2011.228)
35. N. Pantić, M. Spasojević, Ž. Stojanović, Đ. Veljović, J. Krstić, A. M. Balaž, R. Prodanović, O. Prodanović, *J. Polym. Environ.* **30** (2022) 3005 (https://doi.org/10.1007/s10924-021-02364-3)
36. S. Mortazavi, H. Aghaei, *Int. J. Biol. Macromol.* **164** (2020) 1 (https://doi.org/10.1016/j.ijbiomac.2020.07.103).