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COVALENT IMMOBILIZATION OF HORSERADISH PEROXIDASE ONTO MAGNETIC NANOPARTICLES

DIPLOMA THESIS

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KOVALENTNA VEZAVA HRENOVE PEROKSIDAZE NA MAGNETNE NANODELCE

POVZETEK

Biokatalizator hrenova peroksidaza (HRP) je bil uspešno imobiliziran na magnetne nanodelce z metodo aktivacije površine delcev z glutaraldehidom. V študiji so bili raziskani različni parametri, ki vplivajo na postopek imobilizacije encima, kot so vpliv pH-vrednosti medija, ionske moči, koncentracije biokatalizatorja, temperature in koncentracije uporabljenega funkcionalnega aditiva pentaetilenheksanamina. Pri iskanju optimalnih parametrov za uspešno vezavo hrenove peroksidaze na površino magnetnega nosilca smo določevali učinkovitost imobilizacije in aktivnost imobilizirane hrenove peroksidaze. Najvišjo učinkovitost imobilizacije smo zasledili pri pH-vrednosti medija 4.0, le-ta je bila 60 %. Ugotovili smo, da ionska moč ima neposrednega vpliva na imobilizacijo hrenove peroksidaze. Imobilizacija hrenove peroksidaze je bila dosežena pri optimalnih pogojih z relativno aktivnostjo encima, ki je bila 80 %. Imobiliziran encim, dodatno ko-imobiliziran s funkcionalnim aditivom, je pokazal višjo toleranco na vodikov peroksid kot imobilizrana peroksidaza brez dodatka pentaetilenheksanamina.

Ključne besede: magnetni nosilec, s siliko oblečeni maghemitni nanodelci, imobilizacija encima, hrenova peroksidaza, ko-imobilizacija, pentaetilenheksanamin.

COVALENT IMMOBILIZATION OF HORSERADISH PEROXIDASE ONTO MAGNETIC NANOPARTICLES

ABSTRACT

The biocatalyst horseradish peroxidase (HRP) was successfully immobilized onto magnetic nanoparticles by the glutaraldehyde method. The influence of pH medium, ionic strength, the initial protein concentration, temperature and the effect of the functional additive pentaethylenehexanamine on HRP immobilization were studied. The study consisted in seeking for the optimal conditions at which the binding efficiency and the activity of the immobilized horseradish peroxidise were the highest. The results showed that the highest immobilization yield, nearly 60 %, was attained at pH 4.0. It was found that the variation of ionic strength directly affected the HRP immobilization. The enzyme was immobilized at the optimal conditions with the final residual activity of 80 %. However, the enzyme attached onto functionalized magnetic support and additionally co-immobilized with the functional additive, showed better hydrogene peroxide tolerance than the immobilized enzyme without the pentaethylenehexanamine, respectively.

Keywords: magnetic support, silica-coated maghemite nanoparticles, enzyme immobilization, horseradish peroxidase, co-immobilization, pentaethylenehexanamine.

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SYMBOLS AND ABBREVIATIONS

HRP	- horseradish peroxidase
AEAPTS	- 3-(2-aminoethylamino)-propyl-dimethoxymethylsilane
MNPs	- magnetic nanoparticles
RNA	- ribonucleic acid
DNA	- deoxyribunocleic acid
IR	- infrared spectroscopy
MRI	- magnetic resonance image
BSA	- bovine serum albumin
PBS	- phosphate buffer solution
GA	- glutaraldehyde
ISP	- isoelectric point
4-AAP	- 4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one
PEHA	- pentaethylenehexanamine

THEORETICAL PART

1. INTRODUCTION

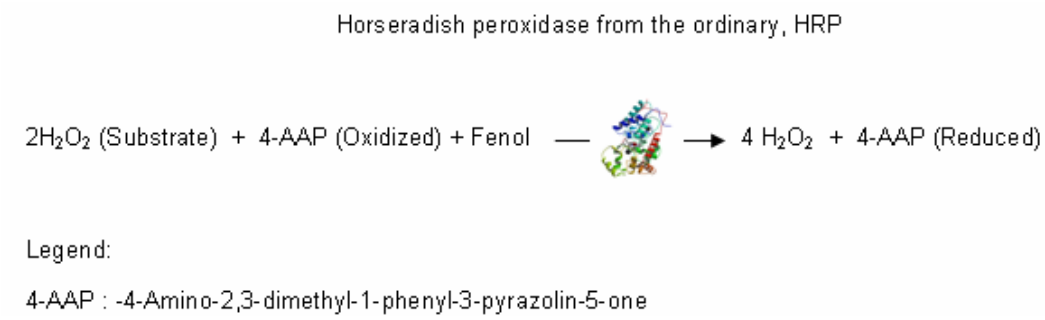
Enzymes are ubiquitous natural biocatalysts of nanometer scale. The potential applications of enzymes are well recognized. Practical use of enzymes has been realized in various industrial processes and products including laundry detergents, and is being expanded in new fields: fine-chemical synthesis, pharmaceuticals, biosensors, bioremediation, biobleaching, polymerase chain reaction, protein digestion in proteomic analysis and biofuel cells. The specificities of enzyme catalysts promise improvements in many applications, but the short lifetimes of enzymes presently limit their usefulness. Improvements in enzyme stability can enable further practical applications. It can reduce the required amount of enzymes, prolong the lifetime of enzyme reactors, increase the potential for enzyme reuse, or maintain the good signal of biosensors. [1]

There have been many approaches to improve the enzyme stability: enzyme immobilization, enzyme modification, protein engineering, and medium engineering. Enzyme immobilization represents the attachment or incorporation of enzyme molecules onto or into large structures, via simple adsorption, covalent attachment, or encapsulation. Enzyme modification is defined by the covalent reactions to the enzyme molecule. Addition of functional groups or polymers on the surface of enzyme molecules may be used to change the surface properties, and result in an improvement of enzyme stability. Protein engineering involves changing the amino acid sequence of an enzyme in order to yield an intrinsically more stable structure using molecular biology techniques such as directed evolution or site-specific mutagenesis. Reaction medium engineering, on the other hand, is to make the enzyme structure more stable by changing the medium around it rather than the enzyme structure. [1]

In recent years, nanostructured materials have been used as supports for enzyme immobilization, since the high surface area: volume ratios of nanoparticles can effectively improve the enzyme loading and the catalytic efficiency of the immobilized enzyme. However, the recovery of nanoparticles immobilized enzyme is often limited. One of the methods is using magnetic nanoparticles. The magnetite-loaded enzymes are easy to recover by a magnetic field, which may optimize operational cost and enhance the product’s purity. [2]

Magnetic supports have been shown wide interest since they have been applied in the fields of bioseparation and biomedicine including protein and enzyme immobilization, immunoassay, RNA and DNA purification, cell isolation and target drug. This technology is based on the immobilization of an affinity ligand on the surface of prefabricated magnetic support and the use of the resulting conjugates for the separation and concentration of biomacromolecules and cells. These magnetic supports can be covalently connected with affinity ligands such as proteins, enzymes and antibodies. However the interactions between affinity ligands and magnetic supports are not quite thoroughly investigated in the literature and the study of protein immobilization onto magnetic supports is important for optimizing conditions for preparation of immuno-magnetic supports with affinity ligands as well as enzyme immobilization. [3]

Horseradish peroxidase (HRP) is an enzyme isolated from horseradish roots, is used extensively in biochemistry applications primarily. HRP is often used in conjugates to determine the presence of a molecular target. HRP is the most desired label for antibodies since it is the smallest and most stable of the three most popular enzyme labels (HRP, alkaline phosphatase, and β–galactosidase). [4]



Scheme 1: Enzymatic oxidation of HRP (EC 1.11.1.10).

Amino groups containing magnetic beads were used in covalent immobilization of the enzyme HRP (EC 1.11.1.10) which is one of a few enzymes that can catalyse the peroxide dependent oxidation of a wide spectrum of organic and inorganic compounds. The magnetic support was derivatized sequentially with glutaraldehyde, and HRP was covalently immobilized on the support via reaction of the amino groups of the enzyme under mild conditions. The effect of various parameters, including pH, enzyme concentration and the addition of a functional additive on the immobilization efficiency of HRP onto dialdehyde activated magnetic nanoparticles was evaluated.

2. THEORETICAL FUNDAMENTS

2.1. Horseradish peroxidase

HRP is isolated from horseradish roots (*Amoracia rusticana*) and belongs to the ferroprotoporphyrin group of peroxidases. HRP is a single chain polypeptide containing four disulfide bridges. It is a glycoprotein containing 18 % carbohydrate. The carbohydrate composition consists of galactose, arabinose, xylose, fucose, mannose, mannosamine, and galactosamine depending upon the specific isozyme. Its molecular weight (44 kDa) includes the polypeptide chain (33,890 Da), hemin plus Ca^{2+} (700 Da), and carbohydrate (9,400 Da). The isoelectric point for HRP isozymes ranges from 3.0 - 9.0. At least seven isozymes of HRP exist. [4]

Figure 2 – 1: Plant source of Peroxidase

The enzyme HRP, found in horseradish, is used extensively in biochemistry applications primarily for its ability to amplify a weak signal and increase detectability of a target molecule.

Chemical structure

The x-ray structural coordinates were scrutinized using the visualization programs RasMol and Protein Structure Explorer. The structural data were analyzed with respect to 1) the length of the α-helices, 2) the number of hydrogen bonds formed in them per residue, and 3) the number of water molecules in hydrogen-bonding distance of up to 3.5 Å per residue. It became immediately apparent that there are basically two groups of α-helices in the molecule: long ones and short ones (Figure 2 – 2). When defining long α-helices as having at least 10 residues, they contribute to 34 % of the secondary structure of HRP. This relates closely to the area contribution of the α-helix amide I IR band at 1660 cm^{-1} (31 %). Shorter α-helices with less than 10 residues contribute to 14 % of the structure of HRP. This seems to indicate that the low-frequency amide I band at 1650 cm^{-1} (20 %) is to a major extent assignable to short helices, whereas the high frequency results mostly from longer helical segments. [5]

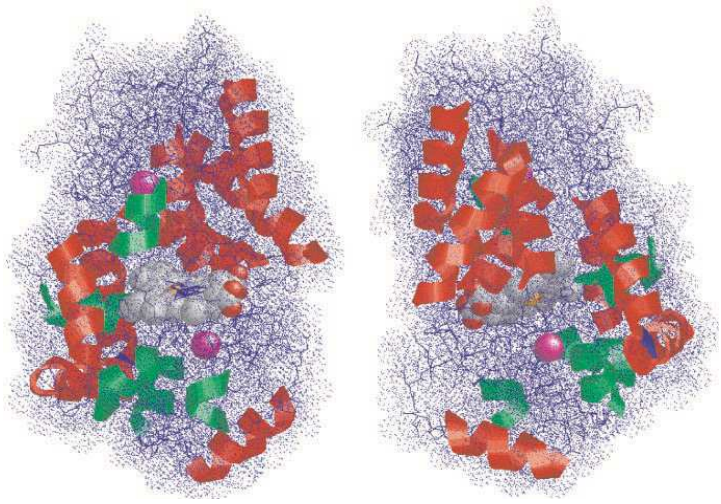


Figure 2 – 2: Display of the tertiary structure of HRP produced with the program RasMol from the atomic coordinates deposited in the Protein Data Bank (Berman et al., 2000) for entry 6ATJ. The locations of the α-helices were determined using the algorithm from Kabsch and Sanders (1983). α-Helices with at least 10 residues are shown in red, shorter ones in green. The heme group and the two Ca^{2+} ions are shown with their van der Waals radii in space-fill mode. To allow for an approximate appreciation of the location of these elements in the molecule, the amino acids are shown with blue lines including their respective van der Waals radii.

Applications

HRP is a glycoprotein with 4 lysine residues for conjugation to a labelled molecule. It produces a coloured, fluorimetric or luminescent derivative of the labelled molecule allowing it to be detected and quantified. HRP is often used in conjugates to determine the presence of a molecular target. [4]

HRP is widely used as a label for immunoglobulins in many different immunochemistry applications including ELISA, immunoblotting and immunohistochemistry. HRP can be conjugated to antibodies by several different methods including glutaraldehyde, periodate oxidation, through disulfide bonds, and also via amino and thiol directed cross–linkers. HRP is the most desired label for antibodies since it is the smallest and most stable of the three most popular enzyme labels (HRP, alkaline phosphatase, and β–galactosidase) and its glycosylation leads to lower non–specific binding. [4]

HRP is ideal in many respects for these applications because it is smaller, more stable and less expensive than other popular alternatives such as alkaline phosphatase. It also has a high turnover rate that allows generation of strong signals in a relatively short time span. [4]

Substrates

Alone, the HRP enzyme, or conjugates thereof, is of little value; its presence must be made visible using a substrate that when oxidized by HRP using hydrogen peroxide as the oxidizing agent, yields a characteristic change that is detectable by spectrophotometric methods. [4]

Numerous substrates for the HRP enzyme have been described commercialized to exploit the desirable features of HRP. These substrates fall into several distinct categories. HRP catalyzes the conversion of chromogenic substrates into colored molecules, and produces light when acting on chemiluminescent substrates (e.g. SuperSignal, ECL). [4]

Characteristics for Native HRP:

a) Extinction coefficient: EmM = 100 when measured at 403 nm.

b) Molecular weight: approx. 44 kDa

c) Isoelectric Point: Isozymes range from 3.0 - 9.0. At least seven isozymes of HRP exist.

d) Substrate Specificity: HRP readily combines with hydrogen peroxide (H_2O_2) and the resultant [HRP–H_2O_2] complex can oxidize a wide variety of chromogenic hydrogen donors. It can also utilize chemiluminescent substrates such as luminol and isoluminol and fluorogenic substrates such as tyramine, homovanillic acid, 4–hydroxyphenyl acetic acid. The Enzyme Explorer's Substrate Index provides links to several chromogenic and chemiluminescent hydrogen donors used to assay peroxidase activity.

e) Inhibitors: The following compounds are inhibitors of HRP: sodium azide, cyanide, L–cystine, dichromate, ethylenethiourea, hydroxylamine, sulfide, vanadate, p–aminobenzoic acid, Cd^{+2}, Co^{+2}, Cu^{+2}, Fe^{+3}, Mn^{+2}, Ni^{+2}, Pb^{+2}.

f) pH Dependence: The pH optimum of HRP is in the range of 6.0 to 6.5; activity at 7.5 is 84 % of the maximum. The enzyme is most stable in the pH range of 5.0 to 9.0.

g) RZ Profile: RZ (Reinheitszahl): the absorbance ratio A_{403}/A_{275}. It is a measure of hemin content of the peroxidase, not enzyme activity. Even preparations with a high RZ value may have low enzymatic activity.

2.2. Magnetic nanoparticles as enzyme support

Nanoparticles are submicron moieties (diameters ranging from 1 to 100 nm according to the used term, although there are examples of nanoparticles several hundreds of nanometers in size) made of inorganic or organic materials, which have many novel properties compared with the bulk materials. In the last decade, nanosize materials have been widely used as support for the immobilization of biomolecules onto insoluble support as an important tool for the fabrication of a diverse range of functional materials of devices. Magnetic nanoparticles are very popular when used in conjunction with biological materials including proteins, peptides, enzymes, antibodies and nucleic acids, because of their unique properties. [6,7,8]

Currently, magnetic nanoparticles are widely studied for their applications in biology and medicine, including magnetic bioseparation and detection of biological entities (cell, protein, nucleic acids, enzyme, bacterials, virus, etc.), clinic diagnosis and therapy, such as MRI (magnetic resonance image), targeted drug delivery and biological labels, RNA and DNA purification, magnetic cell separation and purification and magnetically controlled transport of ante-cancer drugs, as well as hyperthermia generation. These magnetic beads are generally of core-shell type: the biological magnetic core through an organic or polymeric shell. The maghemite particles are preferred because of their grater saturation magnetization. [6,9]

Maghemite (γ-Fe_2O_3) are biocompatible superparamagnetic materials that have low toxicity and strong magnetic properties. They have been widely used for *in* vivo examination including magnetic resonance imaging, contrast enhancement, tissue specific release of therapeutic agents, hyperthermia, magnetic field assisted radionucleide therapy, as well as *in vitro* binding of proteins and enzymes. [7]

Most of the studies with nanoparticles have been dedicated to the improvement of enzyme activity and loading, rather than enzyme stabilization. A recent report using magnetic nanoparticles for the enzyme immobilization is intriguing since a good enzyme stabilization was demonstrated with covalently attached lipase on the magnetic γ-Fe_2O_3 nanoparticles. The final immobilization exhibited high stability for a month, and could be

easily separated from the reaction medium by using a magnetic field. Interestingly, the specific activity (representing enzyme activity per unit mass of enzymes) of covalent-attached lipase on magnetic nanoparticles was lower than that reported for adsorbed enzymes on micrometer-sized polymeric beads. It was argued that the higher activity in the latter case was due to an overestimated enzyme activity since the enzyme desorbs from the beads to the reaction solution, leading to the increase of apparent enzyme activity by the enzyme reaction being carried out in solution rather that in a form of immobilized enzymes. To prevent this effect of leached enzymes on the measurement of enzyme activity, they performed excessive washing after enzyme immobilization, which is important in measuring the unmasked activity of immobilized enzymes. The surface morphology of the magnetic nanoparticles are presented in Figure 2 – 3. [1]

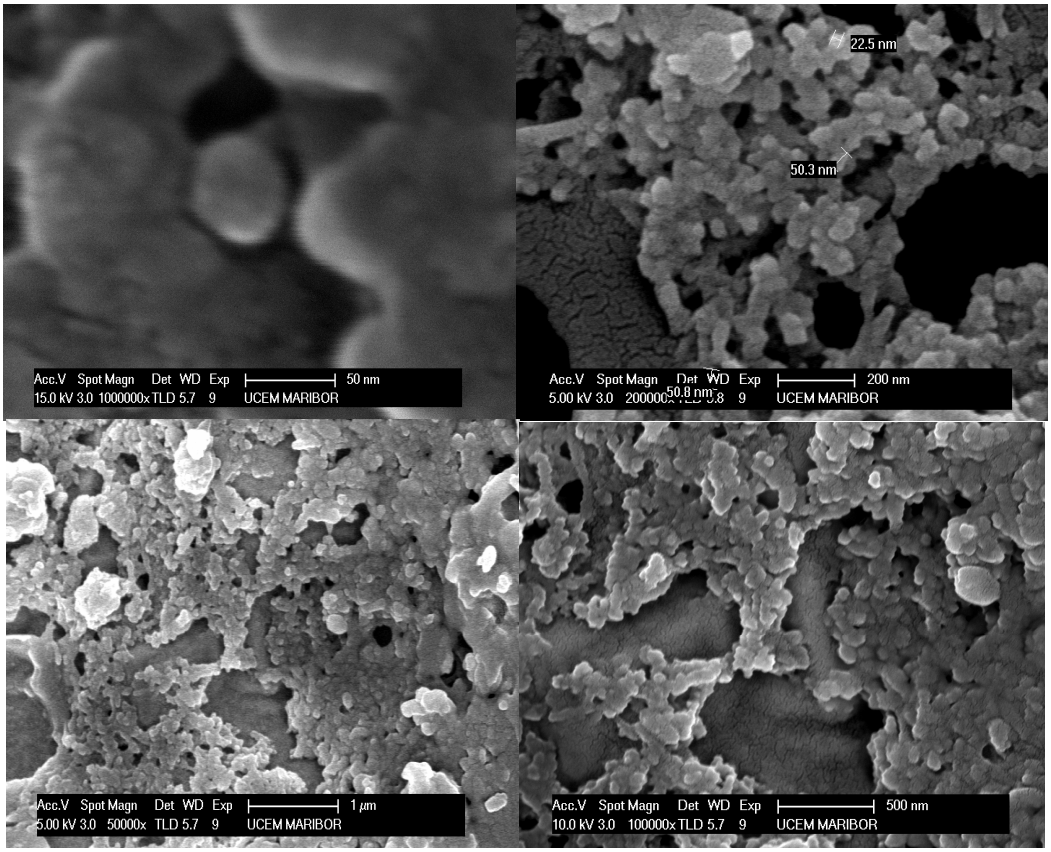


Figure 2 – 3: Scanning Electron Microscopy of magnetic nanoparticles [10].

2.3. Enzyme immobilization

Enzyme immobilization is experiencing an important transition. Combinatorial approaches are increasingly applied in the design of robust immobilized enzymes by rational combination of fundamental immobilization techniques (i.e. non covalent adsorption, covalent binding, entrapment and encapsulation) or with other relevant technologies. [11]

Since the second half of the 20th Century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for various applications. There are several benefits of using immobilized enzymes rather than their soluble counterparts: first, the reusability of heterogeneous biocatalysts with the aim of reducing the production cost by efficient recycling and control of the process; second, as stable and reusable analytic devices for analytic and medical applications; third, as selective adsorbents for purification of proteins and enzymes; fourth, as fundamental tools for solid-phase protein chemistry; and fifth, as effective microdevices for controlled release of protein drugs. [11]

There is no general universally applicable method of enzyme immobilization. The main task is to select a suitable carrier (defined as the non-catalytic part of an immobilized enzyme, on which the catalytic part is constructed), condition (pH, temperature, and nature of medium) and enzyme itself (source, nature and purity) to design an immobilized biocatalyst. The selected method should meet both the catalytic needs (expressed in productivity, space-time yield, stability and selectivity) and the non-catalytic needs (e.g. separation, control, down-streaming process) that are required by a given application. As a result, an immobilized enzyme can be labelled as robust, when both the catalytic and the non-catalytic functions can meet the requirements of a specific application. [11]

It is increasingly appreciated that the availability of a robust immobilized enzyme at an early stage will definitively enable early insight into the process development and save cost not only for then process development but also for the production. However, the lack of guidelines that govern the selection of the immobilization method and the expected performance of the immobilized enzyme for a specific application seriously hampers the application of the rational approach for the design of such robust immobilized enzymes. [11]

The improper strategy of selection for the development of immobilization technique can cause damage to the conformation of biomolecules leading to the deactivation of biomolecular activity. The immobilization method depends on a number of factors, but in

general the method needs to be compatible with the biomolecule being immobilized and the matrix on which immobilization is to proceed. Methods for enzyme immobilization can be classified into three basic categories as follows: [12]

a) Carrier-binding method: the binding of enzymes to water-insoluble carriers such as polysaccharide derivatives, synthetic polymers, porous glass, etc. This is the oldest immobilization technique used for enzymes. In this method the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. When enzymes are immobilized in this way, care is required regarding the selection of carriers as well as in binding techniques. The carrier-binding method can be further sub-classified into three categories according to the binding mode of the enzyme, that is, physical adsorption, ionic binding, and covalent binding. [13]

b) Cross-linking method (covalent binding): intermolecular cross-linking of enzymes by means of bifunctional or multifunctional reagents such as glutaraldehyde, bisdiazobenzidine, hexamethylene diisocyanate, etc. This is based on the binding of functional group of enzymes and matrix via covalent bonds. This method has been employed to improve uniformity, density and distribution of the bound proteins, as well as reproductibility on the surfaces. The conditions for the immobilization by covalent binding are much more complicated than in the case of physical adsorption and entrapment that may alter the conformational structure and active center of an enzyme. The two main precautions which should be taken during covalent attachment involves: the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and the active site of an enzyme must be unaffected by the reagents used. [12,13]

c) Entrapping method: incorporating enzymes into the lattice of a semipermeable gel on enclosing the enzymes in a semipermeable polymer membrane, such as collagen, gelatine cellulose triacetate polyacrylamide, and carrageenan, etc. This is based on confining enzymes in the lattice of a polymer matrix or enclosing enzymes in semipermeable membranes, and it can be classified into the lattice and microcapsule types. This method differs from the covalent binding and cross-linking method in that the enzyme itself does not bind to the gel matrix or membrane. Thus, this method may have wide applicability. However, if a chemical polymerization reaction is used for entrapping, relatively severe conditions are required and lose of enzyme activity occurs in some cases. Therefore, it is necessary to select the most suitable conditions for the immobilization of various enzymes. [13]

EXPERIMENTAL PART

3. MATERIALS AND METHODS

3.1. Enzymes and reagents

HRP (EC 1.11.1.7), with a specific activity expressed in terms of pyrogallol units; one pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20 ℃, was procured from Biozyme Laboratories, UK, whe reas, bovine serum albumin (BSA) was purchased from Sigma-Aldrich, Germany, respectively. All the chemicals used in the present study were generally of reagent grade obtained from commercial sources. Chemicals including amino silane coupling agent (3-(2-aminoethylamino)-propyl-dimethoxymethylsilane or AEAPTS), glutaraldehyde (2.9 % (w/w)) solution, phosphate buffer solution (PBS, 100 mM, ph 7.0) and peroxidase powder (95 %) were of the highest purity and purchased from Sigma-Aldrich Chemicals Company, Germany. The Bradford reagent (Roti-Quant) was obtained from Carl Roth GmbH supplier, Germany. Aqueous solutions were prepared freshly everyday with miliQ water berofe use. The pH values were determined by using a pH meter (Checker, pocked-sized pH meter with replaceable electrode, Hanna Instruments) calibrated with pH 4.0, 7.0 and 14.0 buffers, respectively. The enzyme HRP was stored at -15 ºC until used in the experiments, otherwise, it was kept on ice.

3.2. Analysis and measurements

3.2.1. Instrumentation

Enzyme activity, based on spectrophotometric detection of 4-APP (4-aminoantypyrin), was recorded with the help of Cary 50 Probe UV-visible spectrophotometer. Experiments done on HRP immobilization were supported by a shaker, Heidolph Unimax 1010, respectively. The instruments are presented in Figures 3 – 1 and 3 – 2.

Figure 3 – 1: Shaker Heidolph Unimax 1010.

Figure 3 – 2: Cary 50 Probe UV-visible spectrophotometer.

3.3. Procedures

3.3.1. Synthesis of superparamagnetic amino silane modified silica-coated maghemite nanoparticles

The maghemite nanoparticles were prepared by the thermal co-precipitation reaction. Typically, a solution of mixture of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions was prepared in the presence of a reducing agent, ammonia solution (NH_4OH) on constant stirring. The ideal ratio of Fe^{2+}/Fe^{3+}, for obtaining maximum yield for maghemite nanoparticles during co-precipitation, is 0.5 and needs to be maintained through the

preparation process. The co-precipitation procedure provides a facile and inexpensive method to obtain nano-sized magnetic nanoparticles, either of maghemite or magnetite. Even if the procedure itself is simple, initial temperature and pH of the medium should be carefully controlled, in order to obtain magnetic nanoparticles of desired size and composition. For the experiments to prepare maghemite nanoparticles of uniform size, the initial pH of the iron salts mixture was adjusted to a particular value of 1.5 and kept constant for 30 min. Then, pH was increased rapidly to 11 ($\pm$0.1) by adding 25 % of ammonia solution directly into solution of iron salts and was digested for another 30 min with vigorous stirring. The colour of bulk turned from orange to black immediately. The maghemite nanoparticles were produced in a batch stirred-tank reactor equipped with a mechanical stirrer, as designed in Figure 3 – 3. After the precipitation of the maghemite nanoparticles at the bottom of the reactor, the nanoparticles were washed few minutes with miliQ water by magnetic decantation.

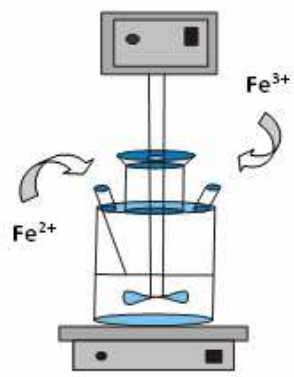


Figure 3 – 3: Batch stirred-tank reactor for the genesis of maghemite (γ-Fe_2O_3) precipitates.

Moreover, surfactant coating procedure was carried out at elevated temperature, 75 ºC for 90 min. In this case, maghemite nanoparticles obtained previously were dispersed in miliQ water and afterwards coated with citric acid (γ=0.5 g ml^{-1}) that served as a surfactant. Later, after surfactant coating, the maghemite nanoparticles were centrifuged (Eppendorf centrifuge 580 LIR) at rotational speed of 3000 rpm for 5 min and the precipitates at the bottom of a centrifuge tube were removed, whereas the magnetic fluid composed of stable maghemite nanoparticles was utilized in the next step of surface functionalization with silica (SiO_2).

In order to obtain a uniformly distributed functional layer of silica onto the surface of maghemite nanoparticles, 200 µL of sodium silicate was consecutively added into magnetic suspension. The reaction was allowed to proceed at 90 ºC for three hours under

continuous mechanical stirring. At the end, the suspension was cooled down and hydrochloric acid was added drop-wise to adjust pH value to 7.0. Finally, the resulted silica-coated maghemite nanoparticles were separated by a permanent magnet (2000 Oe) and dried on air. The particles attained were brown in colour.

Since the mean diameter of the maghemite nanospheres embedded into silica matrix was revealed to be 26 nm, the number of amino silane (AEAPTS) molecules on the surface of every γ-Fe_2O_3 nanoparticles can be obtained by applying the following formula:

$$N_{AEAPS} = \frac{S_{Fe_2O_3/SiO_2}}{S_{AEAPS}} = \frac{4\pi R^2}{S_{AEAPS}} = 5307 \qquad [1]$$

Where $S_{Fe_2O_3/SiO_2}$ refers to the surface area of γ-Fe_2O_3 nanoparticle, S_{AEAPS} is the expected area of surface coverage of about 0.40 nm^2 per amino silane molecule reported in the literature. Thus, silanation reaction by amino silane coupling agent took place in order to provide highly functionalized γ-Fe_2O_3 nanoparticles ready for subsequent surface treatments, the covalent immobilization of HRP. Activation of pendant free amino groups, exposed on the surface of previously chemically modified maghemite nanoparticles, was performed prior the immobilization procedure by the addition of glutaraldehyde with the aid to assure a maximal loading of HRP on the resulting magnetic nanocomposite, depicted in the Figure 3 – 4.

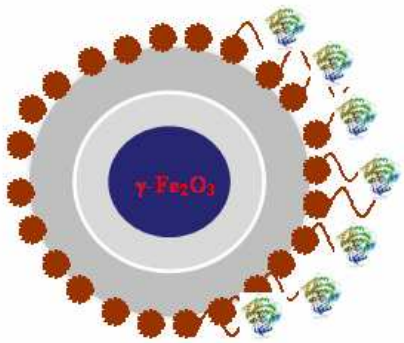


Figure 3 – 4: A schematic illustration of the final hybrid composite based on silica shell/iron core maghemite nanoparticles with the bound enzyme.

3.3.2. Enzyme support activation and immobilization procedure

The γ-Fe_2O_3/SiO_2 nanocomposites, additionally treated with amino silane molecules, were prepared according to the method described above and further utilized for the covalent attachment HRP. Magnetic support of 5 mg was stirred in a solution of 2.9 % (v/v) glutaraldehyde (GA) in phosphate buffer (100 mM, pH 8.0) for two hours. The support activation was carried out by gentle shaking on a thermoshaker (Heidolph Unimax 1010). The activated magnetic supports were then removed in the presence of a magnetic field and rinsed with abundant double-distilled water to remove any excess of GA. Further, the magnetic supports treated with GA were spread onto a clean surface to keep them to dry to completeness. The terminated amino groups on the surface of the magnetic nanostructure react with GA to provide the free terminal aldehyde groups, engaged in the condensation reaction with active amino groups in HRP structure. Then, the activated magnetic supports (5 mg) were added to PBS (100 mM, pH 7.0) containing a certain amount of HRP. Afterwards, the suspension was gently agitated overnight at room temperature. After the completion of each immobilization step, the magnetic nanocomposites with bound enzyme were separated by permanent magnet. The supernatant solution was used to detect the amount of unbound enzyme. The amount of immobilized enzyme onto magnetic supports was determined by measuring the residual amount of enzyme in the supernatant according to Bradford method. The activity of the immobilized HRP was assayed afterwards.

3.3.3. Binding efficiency

The binding efficiency was determined by direct measuring of protein content in the original enzyme solution and the remaining supernatant after immobilization, respectively. For this purpose, amount of HRP immobilized on the maghemite nanoparticles was assayed by a colorimetric method at 595 nm using the Roti-Quant Protein Assay Reagent Concentrate. Here, BSA was used as the protein standard. The amount of the bound HRP on the maghemite nanospheres was calculated as:

$$\varphi[\mu g_{\text{enzyme}} \cdot g^{-1}_{\text{support}}] = \frac{(c_i - c_s) \cdot V_{sample}}{W_{\text{sup } port}} \qquad [2]$$

Where φ is the amount of bound enzyme, c_i and c_s are the corresponding concentrations of the initial enzyme before the immobilization, and residual enzyme in the supernatant solution after immobilization (mg mL^{-1}), V is the volume of the reaction

medium (mL), W is the actual weight of the maghemite support used for immobilization in the present study. All data used in this equation are averages of duplicated experiments.

3.3.4. Enzyme activity assay

The activity of HRP was assayed spectrophotometrically by measuring the increase in absorbance (A_{510}) for a solution of oxidized 4-APP, as it is converted to its reduced form by enzyme HRP. The activity assay was carried out in phosphate buffer (PBS, 100 mM, pH 7.0) containing 4-APP (1.4 mL, 2.5 mM), H_2O_2 (1.4 mL, 1.7 mM), at 25 ºC. The reaction was initiated with addition of 500 µL enzyme solution and was stopped after 10 minutes of assay running.

The same assay medium was used for the determination of the activity of the immobilized HRP. The enzymatic reaction was started by the introduction of HRP immobilized magnetic nanoparticles (5 mg) into the assay medium and was carried out at 25 ºC at ambient conditions. The magnetic nanoparticles carrying immobilized HRP were magnetically concentrated once the reaction completed.

In order to determine enzyme activity, the remaining absorbances of oxidized 4-APP were measured at 510 nm in a variant UV-Vis spectrophotometer.

Activities of HRP attached onto magnetic supports were presented as relative activities expressed in units of percentage, respectively. All activities were correlated to the native enzyme, taken as 100 %.

HRP activity was assayed spectrophotometrically by measuring the initial oxidation rates of 4-AAP, catalyzed by HRP.

The activity of the attached and free HRP was calculated as given by the following mathematical formula:

$$U/mg_{\text{enzyme}} = \frac{\Delta A/\min}{\left[\left(6.58\ M^{-1}cm^{-1}\cdot m_{enzyme}\right)/V_{\text{reaction mixture}}\right]} \qquad [3]$$

Where $\Delta A/\min$ is the slope of the kinetic curve, m_{enzyme} is the mass of enzyme used (free or immobilized) and V the volume of the reaction mixture (3.5 mL).

4. RESULTS AND DISCUSSION

The AEAPTS-treated silica coated magnetic nanoparticles were prepared by conventional co-precipitation reaction of iron oxide. Finally the synthesized magnetic γ-Fe_2O_3/SiO_2-AEAPTS nanoparticles were used to investigate the immobilization procedure of a specific enzyme. HRP was selected as a model protein and covalently immobilized onto magnetic support by the GA method. The influence of different factors on the immobilization binding (efficiency) of HRP onto the GA activated magnetic nanoparticles was studied.

The magnetic nanoparticles (5 mg) were transferred in phosphate buffer (pH 8.0, 1 mL), containing GA solution (2.9 % (w/w)). The reaction was carried out at 25 ºC under constant shaking for 2 h. The activated magnetic support was washed with destilled water. The resulting activated magnetic nanoparticles were further used for the covalent immobilization of HRP.

All HRP immobilization experiments were conducted batchwise at continuous shaking at 25 ℃ for 24 h. In a typical experiment, an appropriate amount of HRP was dissolved in 1 mL of buffer solution of specified pH and 5 mg of GA activated magnetic supports was added afterwards. The mixture was incubated at 25 ℃ for 24 h. Then, the magnetic supports were separated by a permanent magnet. The amount of HRP immobilized onto magnetic supports was determined by measuring the initial and final concentrations of HRP by the Bradford method. The residual protein concentrations in the supernatant and the washing fractions were determined by the Bradford method as well. The activity of the immobilized HRP was assayed afterwards.

4.1. Effect of pH

The immobilization of proteins on the solid supports is usually maximally obtained at the isoelectric point (ISP) of the enzyme. Thus, the effect of the pH value of the medium used in the immobilization procedure of HRP was evaluated. The present work comprised the investigation of the influence of the selected pH medium value on the immobilization process in the range from 3.0 – 9.0.

Figure 4 – 1 presents the immobilization yield of HRP against the medium pH with the initial HRP concentration of 1 mg mL^{-1}. In order to minimize the influence of the buffer, all experiments were carried out in the 0.1 M phosphate buffer. The pH value of the

medium for immobilization was varied from 3.0 to 9.0. Maximum value of immobilization yield was realized at pH value 4.0, which was at the ISP of HRP. The highest immobilization yield was attained at pH 4.0, which corresponds to 60 % of the enzyme HRP immobilized onto chemically modified magnetic nanoparticles. At higher pH values of the medium the immobilization yield dropped drastically.

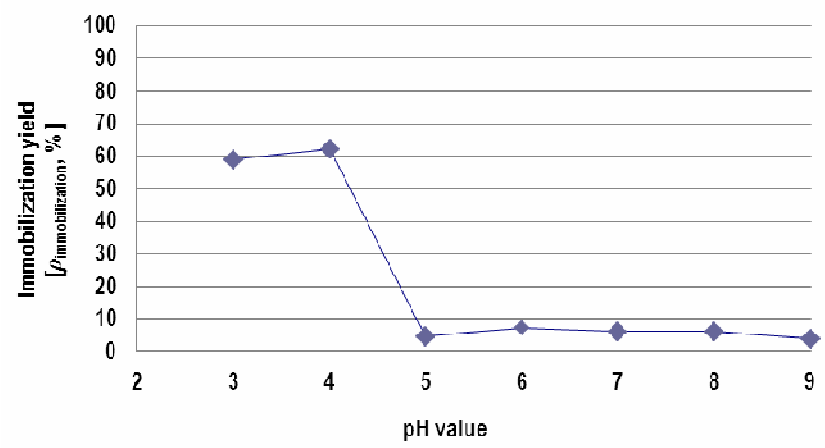


Figure 4 – 1: The effect of pH value on HRP immobilization. The immobilization was carried out at initial HRP concentration of 1 mg mL^{-1} and at 25 ºC.

This result is not unusual, because maximum adsorption of a protein can be accomplished when it has a neutral charge, i.e. at the ISP. At pH different from the ISP, the protein molecules are charged and repel from each other. The neutral original magnetic support surface acquires a positive or negative charge, which prevents the further protein immobilization. At a certain amount of protein molecules on the surface, the process is terminated. Consequently, lower and higher pH values resulted in decreased immobilization yield for HRP.

Figure 4 – 2 shows the results of binding efficiency, expressed in terms of the concentration of immobilized HRP onto magnetic supports.

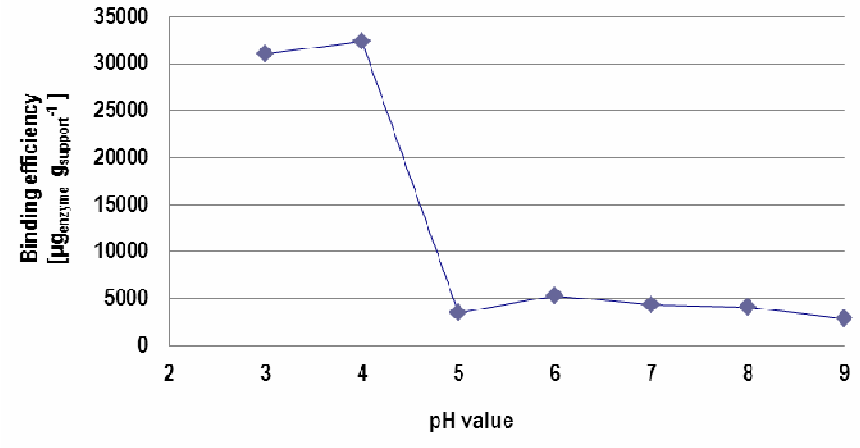


Figure 4 – 2: The effect of pH value on binding efficiency in $\mu g_{enzyme}\ g_{support}^{-1}$. The immobilization was carried out at initial HRP concentration of 1 mg mL^{-1} and at 25 ºC.

As observed in Figure 4 – 2, the highest value of HRP immobilization was achieved at pH 4, which corresponds to 30000 $\mu g_{enzyme}\ g_{support}^{-1}$. The results showed that the maximum value of HRP binding efficiency was obtained in 0.1 M phosphate buffer at pH 4.0.

The residual activity of the immobilized enzyme was assayed afterwards. As shown in Figure 4 – 3, the residual activity decreased at the same time that the pH of the medium used in the immobilization procedure raised from 4.0 to 9.0. Similarly as in the results obtained for binding efficiency, the highest activity of the immobilized enzyme was achieved at pH 4.0, reaching the residual activity of the immobilized HRP of 60 %.

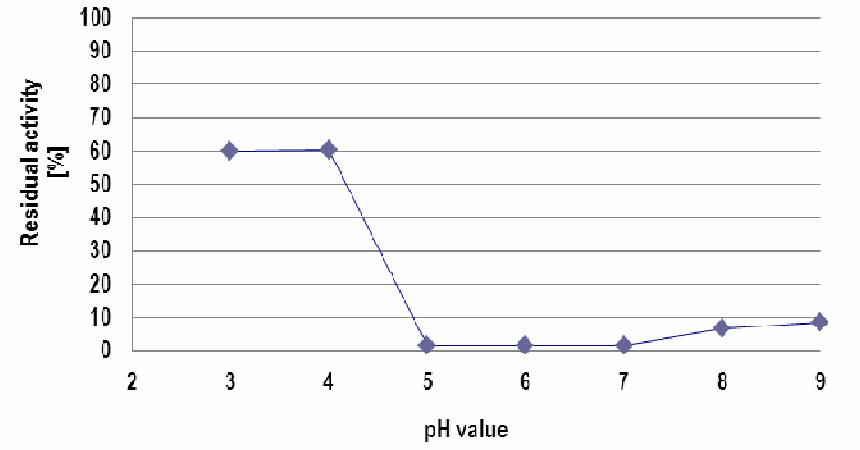


Figure 4 – 3: The effect of pH value on HRP residual activity. The immobilization was carried out at initial HRP concentration of 1 mg mL^{-1} and at 25 ºC.

Figure 4 – 4 shows the estimated concentration of proteins in separate fractions; the concentration of enzyme immobilized onto magnetic nanoparticles and the residual concentration of proteins in the supernatant and the washing fractions, respectively.

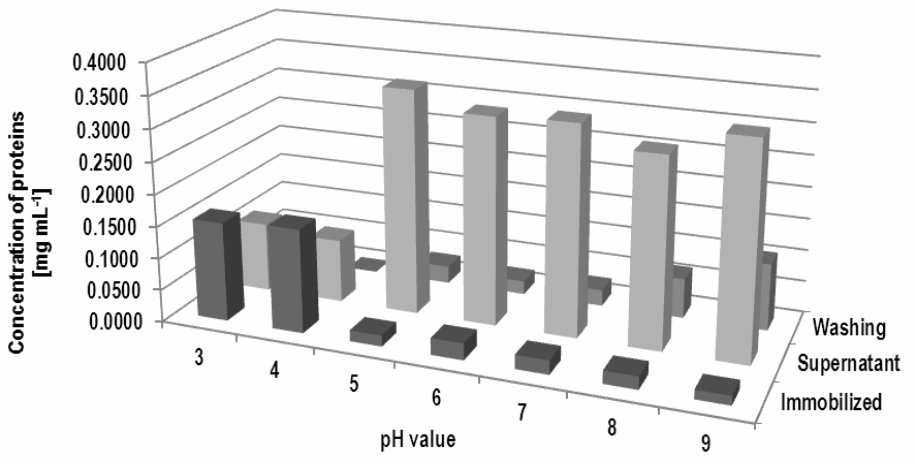


Figure 4 – 4: Concentration of proteins in each fraction after the immobilization procedure. The immobilization was carried out at initial HRP concentration of 1 mg mL^{-1} and at 25 ºC.

In the pH range between 3.0 and 4.0 the enzyme was successfully bound onto magnetic nanoparticles, as the immobilization yield of HRP onto magnetic support was the highest in the comparison to other pH values. The remaining concentration of proteins found in the supernatant and the washing fractions were higher at increasing the pH values, indicating the unsuccessful binding of HRP.

Figure 4 – 5 shows the estimated residual activity in each fraction upon the completion of immobilization of HRP.

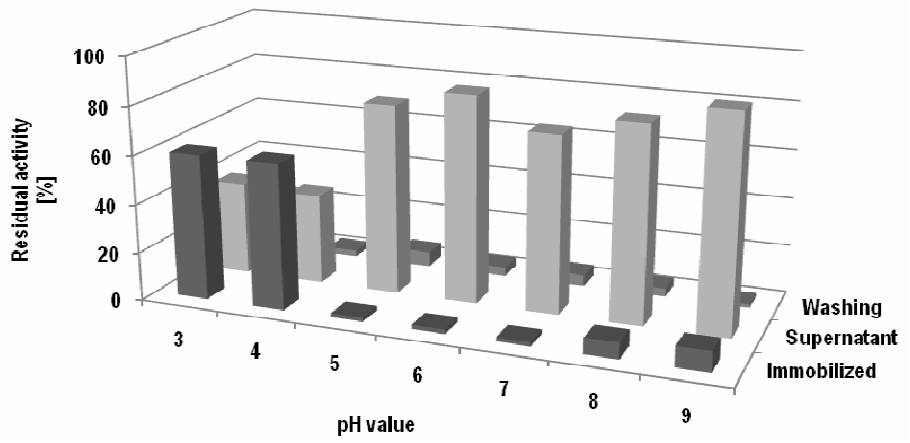


Figure 4 – 5: The residual activity of the enzyme after the immobilization procedure. The immobilization was carried out at initial HRP concentration of 1 mg mL^{-1} and at 25 ºC.

Nevertheless, the residual activity of different fractions after the immobilization was estimated in order to verify the activity of the bound and unbound enzyme. As shown, in the Figure 4 – 3, the immobilized HRP in the pH range from 3.0 to 4.0 showed a remarkably high activity. At increasing the pH value, the binding of HRP onto magnetic nanoparticles was substantially low, resulting in leaking of the enzyme in the supernatant and washing fractions. Therefore, the residual activity of unbound HRP increased in the remaining fractions after the immobilization, thus, at higher pH values from 5.0 – 9.0. In comparison to supernatant fractions, the washing fractions contained negligible amount of proteins as the estimated residual activity was low. On these grounds, we presume that the enzyme HRP was enough stable after rinsing the magnetic support with appropriate buffer solution.

The results on the effect of the pH value of the medium on enzyme immobilization showed that the best pH medium value for the immobilization of HRP was 4.0, for the amount of enzyme attached onto the chemically modified magnetic nanoparticles was higher in comparison to other pH values tested.

4.2. Effect of ionic strength

In order to find out the effect of the ionic strength on the protein immobilization, the immobilization of HRP was studied in buffer solution with the addition of salt, sodium chloride (NaCl). The initial HRP concentration was 1 mg mL^{-1}. The concentration of NaCl was varied from 0.0 to 1.0 M. All the experiments were carried out with 5 mg of maghemite nanoparticles as enzyme support. All experiments were carried out in 0.1 M phosphate buffer at pH 4.0, respectively. The results of immobilization yield are summarized in Figure 4 – 6.

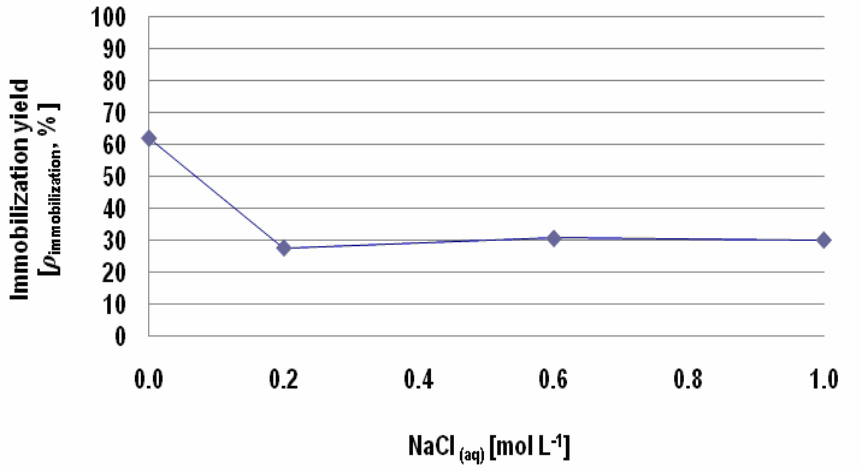


Figure 4 – 6: The effect of ionic strength on HRP immobilization yield. The immobilization trials were made with the initial concentration of HRP of 1 mg mL^{-1} in a buffer solution at pH 4.0 and at 25 ºC.

Apparently, it was found that the presence of salt, NaCl, affected the HRP immobilization. As shown in Figure 4 – 6, the highest immobilization yield was attained, when no salt was added during immobilization.

As observed in Figure 4 – 7 the binding efficiency in terms of the amount of enzyme immobilized onto magnetic nanoparticles was estimated as well.

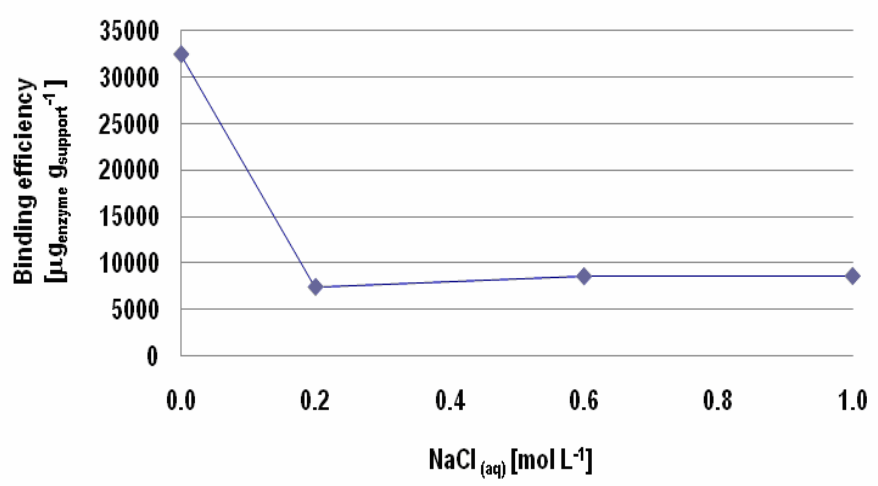


Figure 4 – 7: The effect of ionic strength on HRP binding efficiency. The immobilization was carried out with the initial concentration of HRP of 1 mg mL^{-1} in a buffer solution at pH 4.0 and at 25 ºC.

However, the amount of immobilized HRP substantially decreased with the increase of the salt concentration. Salt concentration, increased from 0.0 to 1.0 M, caused no enhancement on the binding efficiency of HRP. Therefore, it can be concluded, that the

presence of salt significantly affected the immobilization process. Apparently, the addition of salt with a concentration of NaCl higher than 0.2 mol L^{-1} gave poor immobilization yields, ranging from 28 % to 30 %.

The Figure 4 – 8 shows the estimated residual activity of the immobilized enzyme at different concentrations of salt used in the study.

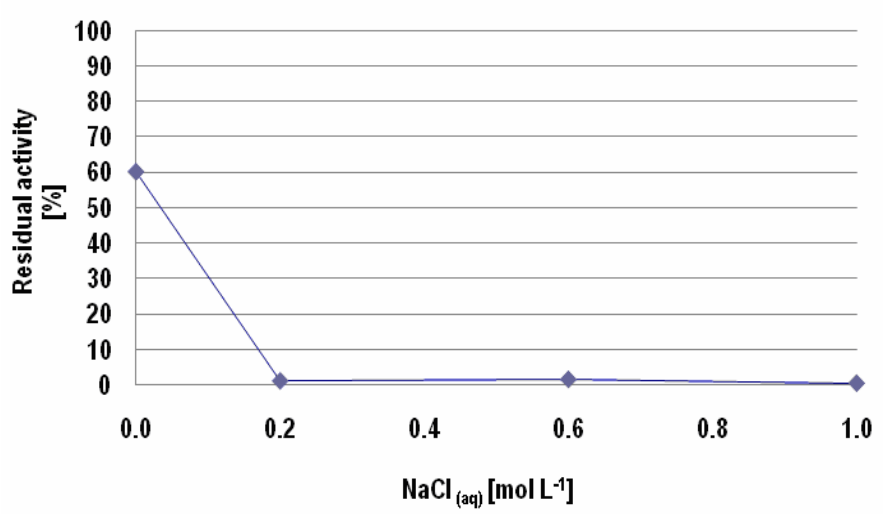


Figure 4 – 8: The effect of ionic strength on HRP residual activity. The immobilization trials were conducted with the initial HRP concentration of 1 mg mL^{-1} in a buffer solution at pH 4.0 and at 25 ºC.

In presented in Figure 4 – 8, the residual activity of the immobilized enzyme was found to be lower in trials, when the salt solution was added. In the absence of salt, the residual activity achieved the highest value, corresponding to 60 % of activity of the immobilized HRP in comparison to its native counterpart.

In Figure 4 – 9, we can observe the remaining concentration of proteins in the fractions of supernatant and washing at different salt concentrations.

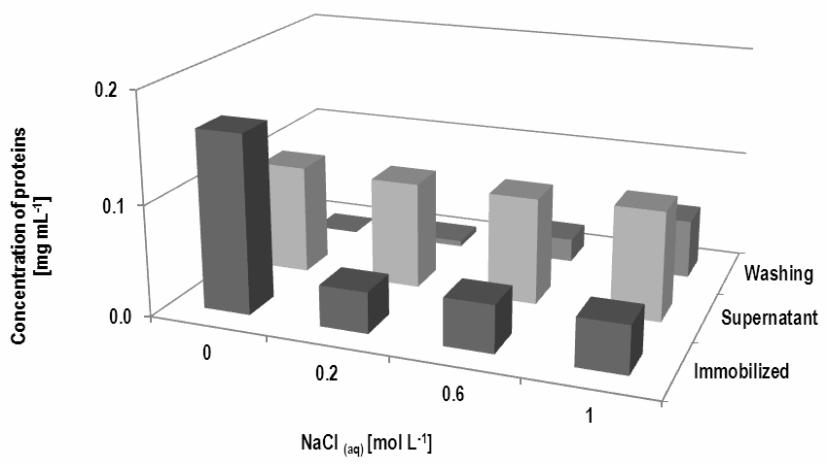


Figure 4 – 9: The concentration of proteins in different fractions upon the immobilization process of the enzyme HRP. The immobilization trials were carried out with the initial HRP concentration of 1 mg mL^{-1} in a buffer solution at pH 4.0 and at 25 ºC.

However, as shown in Figure 4 – 9, the concentration of the immobilized enzyme onto magnetic nanoparticles was recognizably higher only in the case where no salt was added. Consequently, the resulting immobilization yield was the highest as well in the absence of sodium chloride solution. On the other hand, the remaining concentration of proteins estimated in the supernatant and washing fractions increased with increasing the ionic strength during immobilization, indicating the unsuccessful binding of HRP at higher salt concentrations. The residual activity in the different fractions upon immobilization of HRP are shown in Figure 4 – 10.

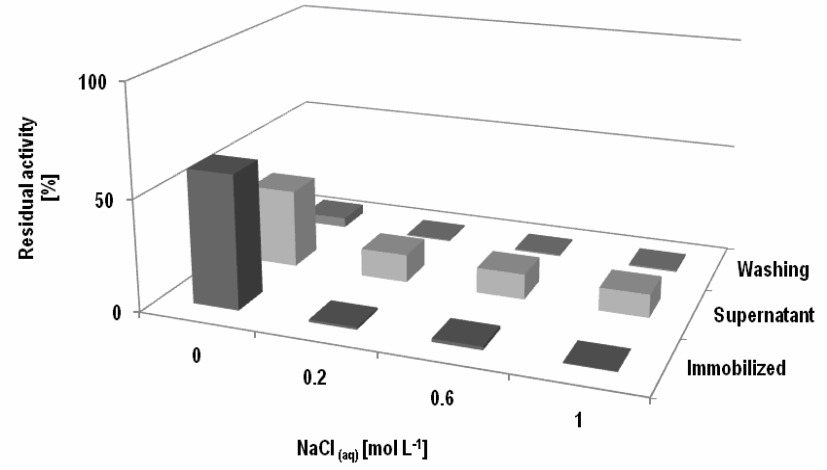


Figure 4 – 10: The residual activity of HRP enzyme in different fractions after the immobilization procedure at the different salt concentrations. The immobilization trials were conducted with the initial HRP concentration of 1 mg mL^{-1} in a buffer solution at pH 4.0 and at 25 ºC.

Apparently, the immobilized HRP onto magnetic nanoparticles retained its highest activity in phosphate 0.1 M buffer solution at pH 4.0 and in the absence of salt. The HRP binding efficiency decreased with the increase of NaCl concentration. The corresponding activity of the immobilized HRP decreased with the addition of salt solution during immobilization as well.

Maximum residual activity was optimal at the pH 4.0, where, we presume that the enzyme has neutral charge and minimum solubility. Apparently, the addition of salt has obvious influence on the protein charge and solubility. Therefore, it can be concluded that the presence of salt affected the immobilization process.

4.3. Effect of enzyme concentration

In order to find the effect of enzyme concentration on the immobilization process different initial concentrations of HRP were tested. Additionally, in order to minimize the influence of buffer solution as medium for immobilization, all experiments were carried out with 5 mg of maghemite nanoparticles as enzyme support, in 0.1 M phosphate buffer at pH 4.0, respectively. Thus, different amounts of HRP enzyme were attempted to be immobilized onto functionalized magnetic nanoparticles. The enzyme concentration range expanded from 5 to 800 μg mL^{-1}.

Figure 4 – 11 presents the results of immobilization yield at different enzyme concentrations. The complete binding of HRP onto magnetic nanoparticles was achieved at the enzyme concentrations ranging from 50 μg ml^{-1} to 200 μg ml^{-1}. In this regards, the immobilization yield was 100 %, meaning that the immobilization yield reached its maximum value.

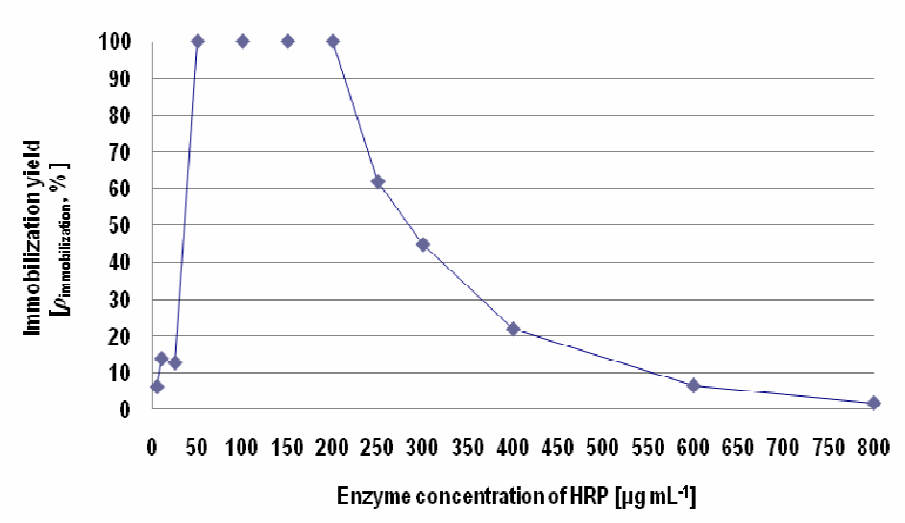


Figure 4 – 11: The effect of enzyme concentration on immobilization yield. The immobilization was accomplished in a buffer solution at pH 4.0 and at 25 ºC.

As shown in Figure 4 – 11, the immobilization yield increased with increasing the enzyme concentration up to concentration of HRP of 50 µg ml^{-1}. Afterwards, in the enzyme concentration range from 50 to 200 µg ml^{-1} the immobilization yield remained unvaried. At the concentration higher than 200 µg ml^{-1} the immobilization yield started to decrease gradually.

Figure 4 – 12 shows the amount of immobilized HRP onto magnetic nanoparticles when different enzyme concentrations were used.

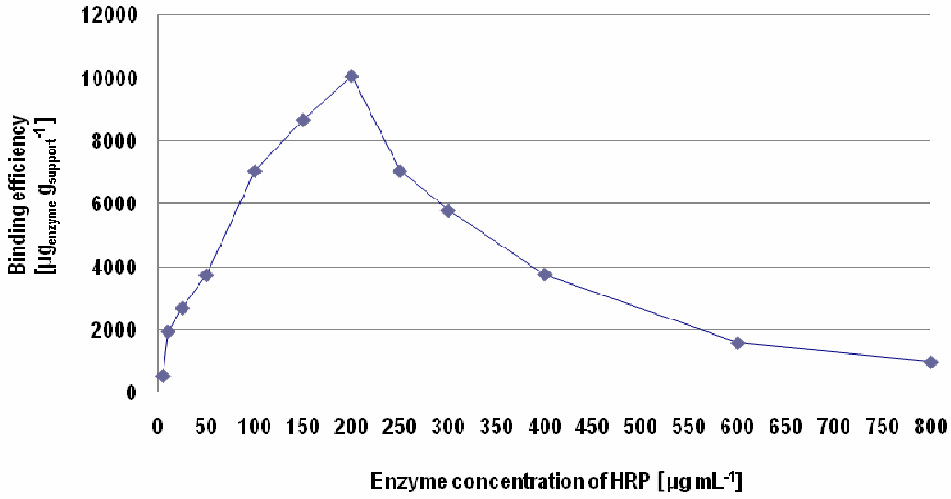


Figure 4 – 12: The effect of enzyme concentration on binding efficiency expressed in $\mu g_{enzyme}\ g_{support}^{-1}$. The immobilization was carried out in a buffer solution at pH 4.0 and at 25 ºC.

The results presented in Figure 4 – 12 are in complete accordance with results obtained previously, showing that the amount of the immobilized HRP increased at the same time as HRP concentration increased. A complete immobilization of enzyme onto magnetic nanoparticles surface was accomplished in the enzyme concentration range from 50 to 200 μg ml^{-1}. In case when lower concentration of enzyme was used, only small amounts of HRP were successfully bound onto magnetic supports. The maximum binding efficiency (10000 $\mu g_{enzyme}\ g_{support}^{-1}$) was achieved when the HRP concentration did not exceed the concentration value of 200 μg ml^{-1}. At higher enzyme concentrations the amount of immobilized HRP decreased consistently.

The residual activity of the immobilized HRP was checked afterwards. As shown in the Figure 4 – 13, the highest residual activity was achieved when 250 μg ml^{-1} of enzyme concentration was attempted to be immobilized. The residual activity of the immobilized HRP was 2.5 %.

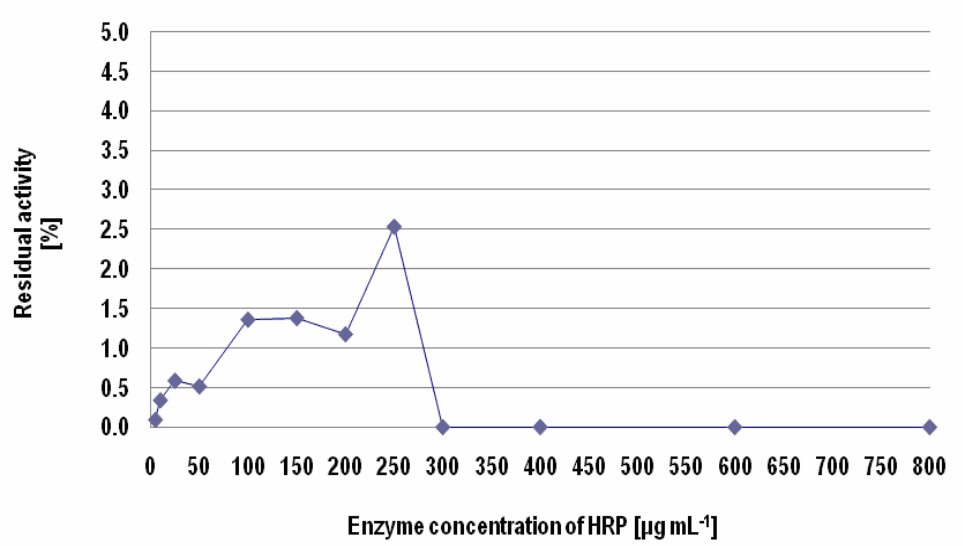


Figure 4 – 13: The effect of enzyme concentration on residual activity.

As observed in Figure 4 – 13, residual activity increased with increasing the HRP concentration. At the enzyme concentration higher then 250 μg mL^{-1} the immobilized enzyme did not show any activity, respectively.

In Figure 4 – 14, we can observe the estimated protein concentration in separate fractions; the concentration of enzyme immobilized onto magnetic nanoparticles and the residual concentration of proteins in the supernatant and the washing fractions.

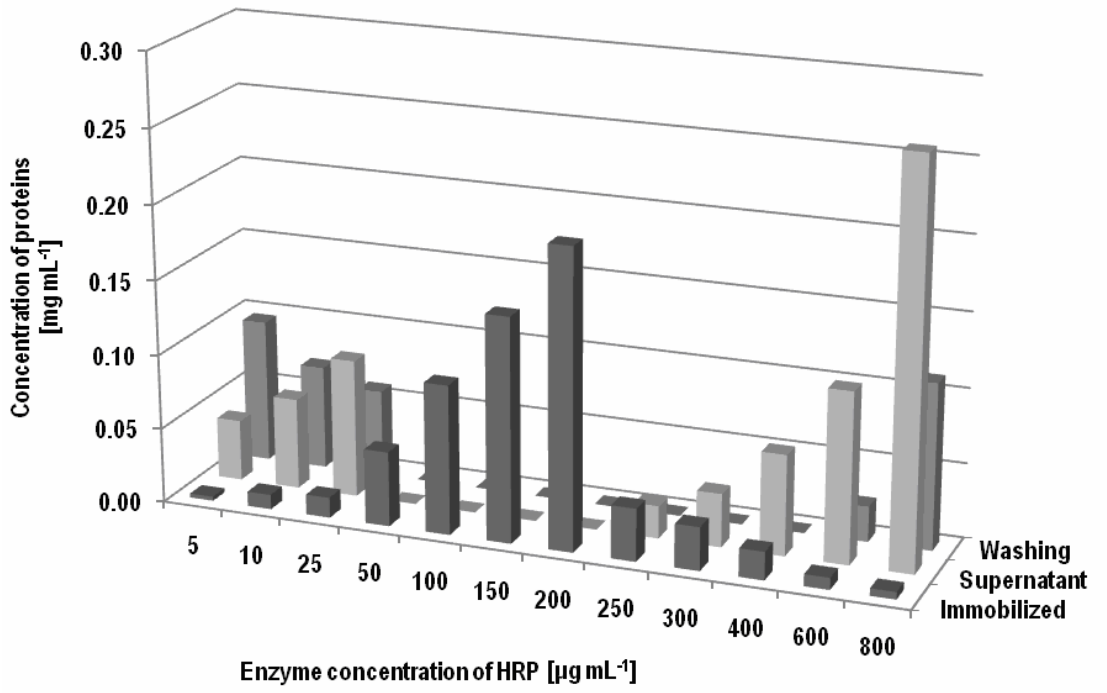


Figure 4 – 14: The concentration of proteins in different fractions after the immobilization procedure.

The concentration of the immobilized enzyme onto magnetic nanoparticles increased with increasing the enzyme concentration for immobilization. Obviously, higher enzyme concentrations facilitated the immobilization procedure, resulting in higher binding efficiencies obtained when higher enzyme concentrations were applied. On the contrary, the remaining concentration of proteins that corresponds to unbound HRP enzyme was found to be high in the enzyme concentration ranges from 5 to 25 µg mL^{-1} and from 250 to 800 µg mL^{-1}. At the HRP concentrations, at which the immobilization yield was maximally achieved, we could not detect any traces of unbound HRP in the supernatant and washing fractions, respectively.

Figure 4 – 15 shows residual activity of the bound HRP in comparison to those found in the fractions of supernatants and washings.

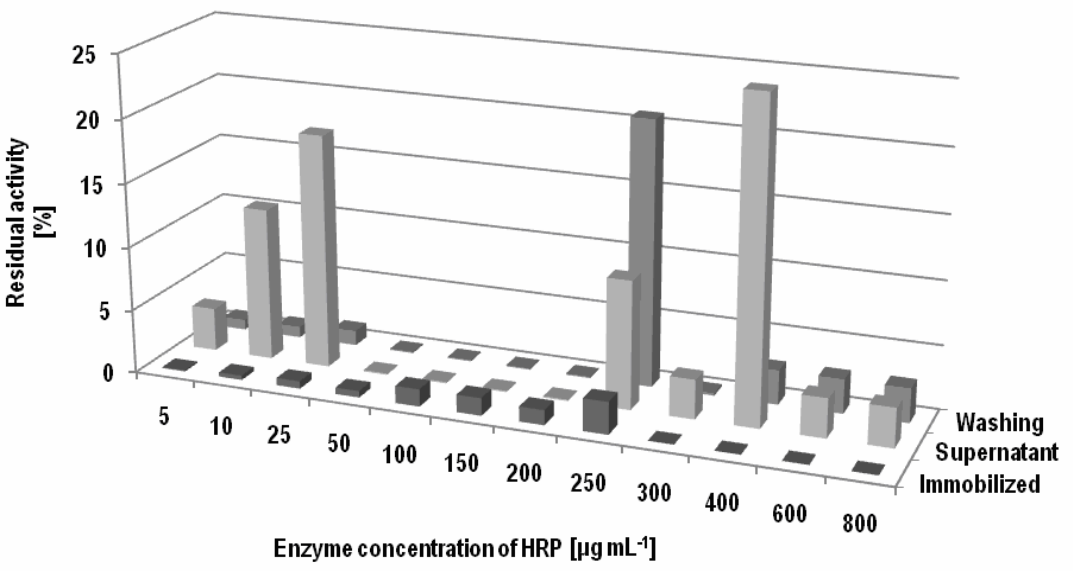


Figure 4 – 15: Residual activity of enzyme in different fractions after the immobilization procedure at different enzyme concentrations.

Apparently, some enzyme activity was found in the supernatant and the washing fractions. This was valid only when the enzyme concentration was extremely low or high, respectively. It could be deduced that at those enzyme concentrations the enzyme was not successfully immobilized favouring its leaking into the supernatant and washing fractions. The successful binding of HRP enzyme was achieved in the concentration range from 50 to 200 µg mL^{-1}, in which the immobilized enzyme showed residual activity equivalent to 1.4 % in comparison to its native counterpart. As the immobilization yield was 100 % in the enzyme concentration range from 50 to 200 µg ml^{-1}, we presume that the enzyme acquired a higher stability in comparison to other concentrations used.

4.4. Effect of functional additive

The effect of the functional additive was studied with the addition of pentaethylenehexanamine (PEHA). PEHA was co-immobilized with HRP enzyme at different mixing times.

PEHA has important applications in a number of industries. It is a hardener used with epoxy resins that have both industrial and consumer applications. It is an intermediate in the synthesis of several substances, e.g. chemicals that are mixed with asphalt to pave roads. PEHA has widespread use in the manufacture of lubricating oil and fuel additives. Although workers would be expected to be the population primarily exposed

to PEHA, consumer exposure cannot be ruled out. Because PEHA may produce long-term effects in aquatic ecosystems, its release in waste streams is also of concern. [15, 16]

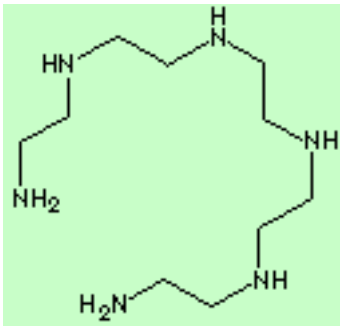


Figure 4 – 16: Pentaethylenehexanamine. [17]

The modest improvements in binding efficiency and residual activity when using bare enzyme for immobilization prompted us to investigate the addition of PEHA as a functional additive, which has also been used as amine donor in enzyme immobilization techniques.

Therefore, in order to study the effect of PEHA on HRP immobilization, different parameters as the concentration of PEHA and the mixing time were studied. The concentration of PEHA added during the process of co-immobilization with the native HRP varied from 5 mmol L^{-1} to 20 mmol L^{-1}. The concentration of PEHA in a stock solution was maintained at 100 mM at pH 6.4.

Next, the pre-treatment of HRP enzyme with the functional additive PEHA was carried out at different mixing times in the range from 15 to 60 min.

In Figure 4 – 17, the results of immobilization yield at different mixing times and concentrations of PEHA are presented.

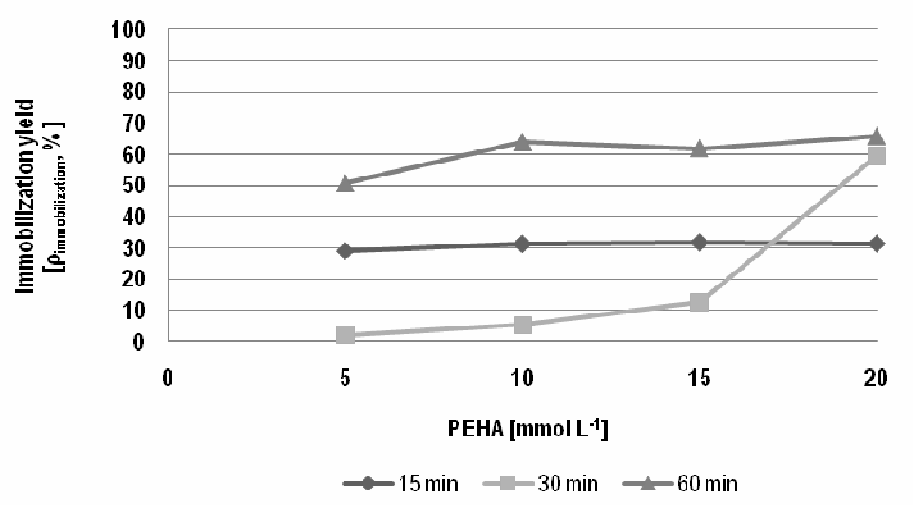


Figure 4 – 17: The effect of the concentration of PEHA on immobilization yield after 15, 30 and 60 min of incubation. The immobilization procedure was performed in a buffer solution at pH 4.0 and with the initial HRP concentration of 250 μg mL^{-1} at 25 ºC.

It was found out that when the concentration of PEHA increased, the percentage of the immobilization yield increased afterwards. This observation was also noticed when different times of incubation were applied. The resulting immobilization yield was nearly 30 % when the time of pre-treatment of HRP with PEHA lasted for 15 min. Moreover, the immobilization yield after 15 min of incubation remained unvaried with the concentration of PEHA. Thus, the resulting immobilization yield was estimated to be in the range from 29 % to 32 % in dependence to the concentration range of PEHA from 5 to 20 mmol L^{-1}. On the other hand, the immobilization yield resulted to be higher for the mixing times of 30 and 60 min and at the concentration of PEHA of 20 mmol L^{-1}. At the concentration of PEHA of 20 mmol L^{-1} and at the mixing time of 30 min the immobilization yield was 60 %. On the contrary, the immobilization yield at the same PEHA concentration and at the mixing time of 60 min was 65 %. Apparently, as shown in the figure, the immobilization yield at the mixing time of 60 min was higher in the whole concentration range of PEHA in comparison to other mixing times of 15 and 30 min.

Residual activity of the immobilized HRP, co-immobilized with different concentrations of PEHA, was tested afterwards. Similarly, as shown in Figure 4 – 18, the residual activity of immobilized HRP increased as the concentration of PEHA increased as well.

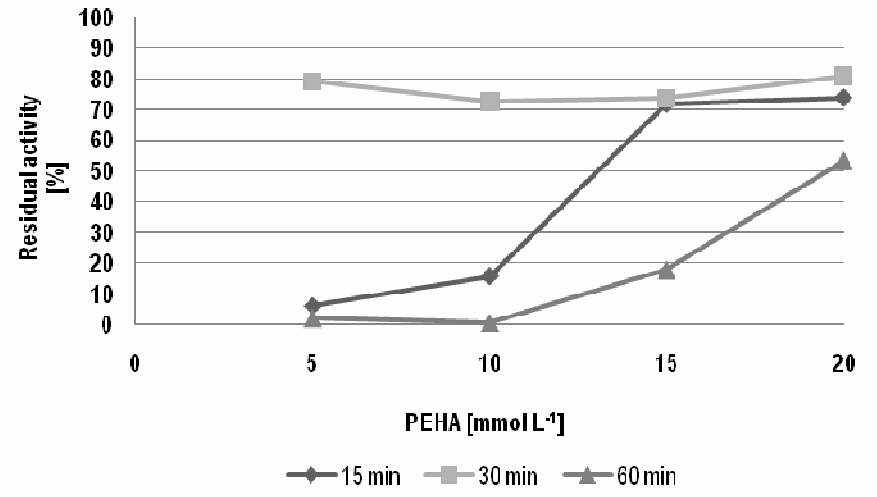


Figure 4 – 18: The effect of concentration of PEHA on residual activity after the period of incubation for 15, 30 and 60 min. The immobilization was performed in buffer solution at pH 4.0 and at the initial HRP concentration of 250 μg mL^{-1} at 25 ºC.

As observed, the highest residual activity was achieved when 20 mmol L^{-1} of PEHA was used and when the time of incubation lasted for 30 min. The resulting residual activity was 80 %. In the case of 15 min of incubation the residual activity was found to be 72 %, whereas in the case of 60 min of pre-treatment time the immobilized HRP retained 53 % of its activity, respectively. The immobilized HRP, when the time of incubation was 30 min, maintained high activity independent to the concentration of PEHA. In the case of 15 and 60 min of enzyme incubation with PEHA, the residual activity of the immobilized HRP dropped significantly when the concentration of PEHA decreased. At the concentration of PEHA of 5 mmol L^{-1} the residual activities of the immobilized HRP at the mixing times of 15 and 60 min were 6 % and 2 %, respectively.

Apparently, the immobilized HRP improved its stability when PEHA was introduced into the immobilization of HRP.

In conclusion, the optimal concentration of PEHA was 20 mmol L^{-1}, respectively. The time of incubation needed for the pre-treatment of the surface of the soluble HRP was found to be optimal at 30 min.

4.5. Effect of temperature

It is well known that moderately high temperatures may favour the vibration of enzyme and support, increasing the possibilities of getting more enzyme-support linkages.

In order to find out the effect of temperature on the enzyme immobilization, the immobilization of HRP, co-immobilized with PEHA, was conducted at various temperatures. The temperature range varied from 20 to 45 ºC, respectively. The immobilization procedure for HRP was performed in a buffer solution at pH 4.0 and with the enzyme concentration of 250 μg mL^{-1}. The enzyme was additionally co-immobilized with PEHA (20 mmol L^{-1}) with a duration of co-immobilization process of 30 min. All the experiments were carried out with 5 mg of maghemite nanoparticles as enzyme support. The results are summarized in Figure 4 – 19.

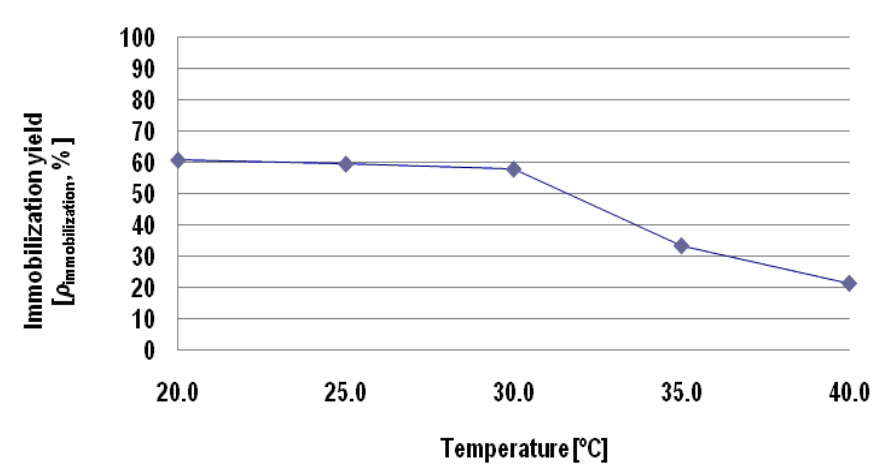


Figure 4 – 19: The effect of temperature on immobilization yield. The immobilization was performed in a buffer solution at pH 4.0 with the initial HRP concentration of 250 μg mL^{-1} and PEHA (20 mmol L^{-1}).

The Figure 4 – 19 clearly shows that the immobilization yield decreased with increasing the temperature. Thus, we presumed, that higher temperatures do not favour the immobilization process, as the immobilization yield, in comparison to lower temperatures, gradually decreased. No significant change in the immobilization yield was observed at the tested temperature from 20 to 30 ºC, indicating that the binding of HRP was highly maintained at moderately lower temperatures. Thus, the optimal temperature for immobilization was found to be at 25 ºC.

In the Figure 4 – 20 the amount of HRP bound onto magnetic nanoparticles against the temperature is presented. The binding efficiency is expressed in terms of $\mu g_{enzyme}\ g_{support}^{-1}$.

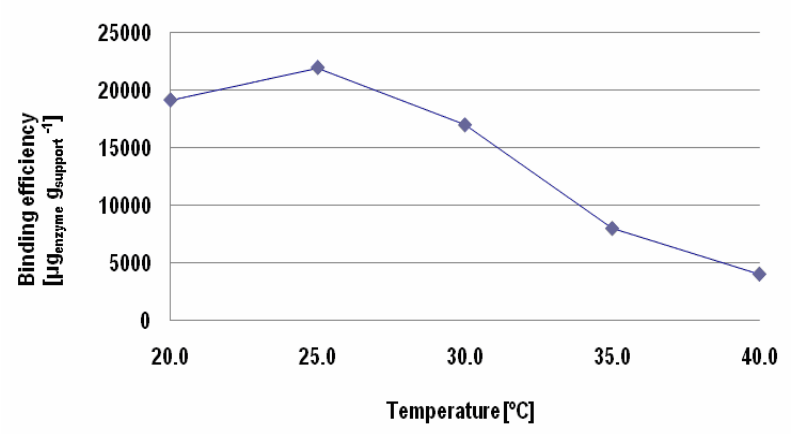


Figure 4 – 20: The effect of temperature on binding efficiency in $\mu g_{enzyme}\ g_{support}^{-1}$.

As previously mentioned, the optimal binding efficiency was obtained at the temperature of 25 ºC. The binding capacity at the temperature 25 ºC resulted in 22000 $\mu g_{enzyme}\ g_{support}^{-1}$, whereas at the temperature 20 ºC decreased to the value of 20000 $\mu g_{enzyme}\ g_{support}^{-1}$. At the temperature 40 ºC the immobilization yield was only 4000 $\mu g_{enzyme}\ g_{support}^{-1}$, respectively.

The effect of temperature (20 – 40 ºC) on the residual activity is shown in Figure 4 – 21.

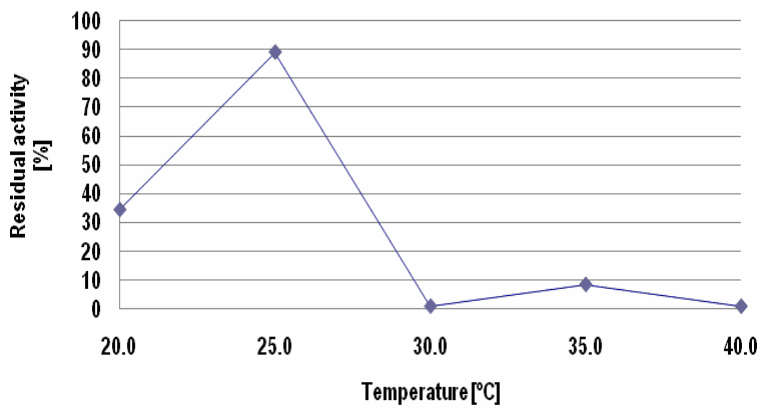


Figure 4 – 21: The effect of temperature on residual activity.

In the case for the immobilized enzyme, the residual activity of the enzyme dropped with increasing the temperature. The maximum residual activity achieved in the investigation of the effect of temperature on the immobilization process was close to 90 %. At 20 ºC, the residual activity of the immobilized HRP was 35 % and at higher

temperatures the residual activity was even lower, not exceeding 10 % of its relative activity.

Figure 4 – 22 presents the concentration of proteins in different fractions obtained after the immobilization procedure.

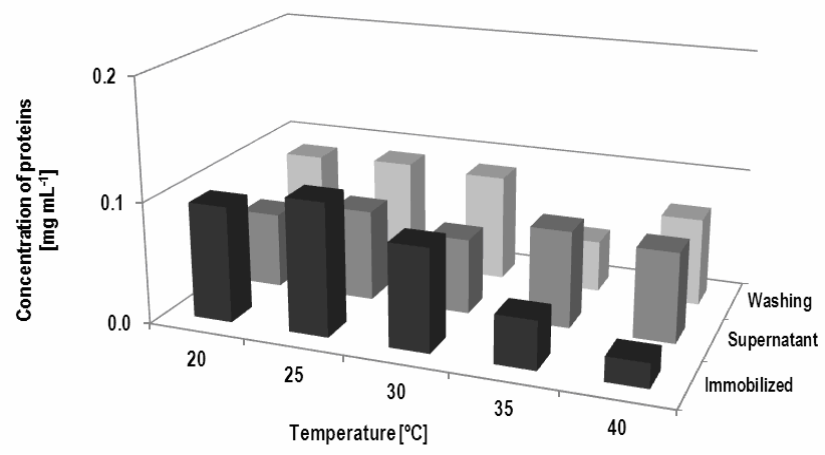


Figure 4 – 22: Concentration of proteins in different fractions upon immobilization process.

The highest concentration of bound enzyme was achieved at 25 ºC. At other temperatures the binding of HRP was less successful, favouring its leaking into the supernatant and washing fractions. As concluded, the concentration of immobilized HRP decreased with rising the temperature.

The residual activity in the remaining fractions upon immobilization are summarized in the Figure 4 – 23.

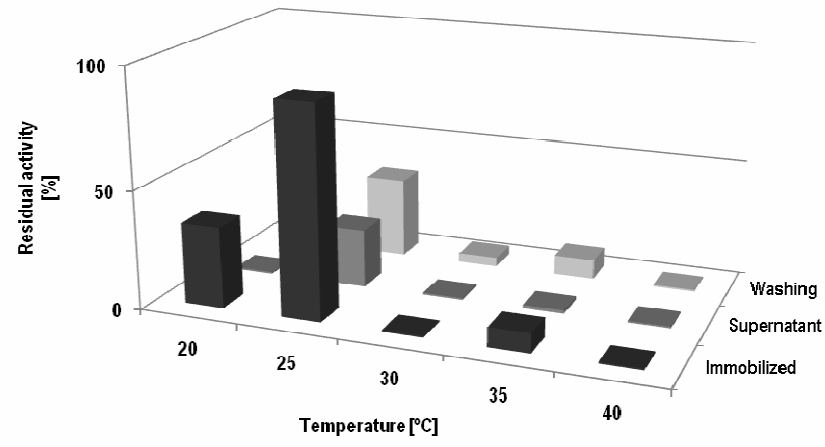


Figure 4 – 23: The remaining residual activity in different fractions upon immobilization procedure.

A similar attitude was observed when residual activity was tested. Apparently, the immobilized HRP retained its highest activity at the temperature 25 ºC that corresponds to 90 % of its relative activity. As already shown above, the binding efficiency decreased with increasing the temperature, thus, the corresponding activity of the immobilized HRP decreased in a similar vain. Apparently, some activity was also found in the supernatant and washing fractions in the whole temperature range. The highest values of residual activity were obtained at the temperature 25 ºC.

On the basis of the results obtained, we can conclude that the optimal temperature for immobilization of HRP was at 25 ºC.

4.6. Hydrogen peroxide tolerance

Peroxidases are metabolizing enzymes that are distributed in fungal, plant, and other organisms. The enzymes are involved in important physiological roles such as biosynthesis of hormones and stress and pathogen responses via the oxidation of various substrates at the expense of H_2O_2. The first step of the common catalytic sequence of peroxidases is the reaction between the ferric resting enzyme and H_2O_2. [19]

As mentioned above, one of the limitations of HRP is its low stability towards H_2O_2 owing to the oxidation of the poryphyrin ring, presumably by reactive species such as superoxide anions, hydroxyl radicals and singlet oxygen produced during the catalytic cycle. [20]

In order to investigate the H_2O_2 tolerance we tested the activity at different H_2O_2 concentrations using free HRP and bound HRP. The hydrogen peroxide tolerance was repeated also with the bound HRP with co-immobilized functional additive PEHA. Concentrations of H_2O_2 were between 50 μM and 80 mM. The enzyme HRP was first preincubated for 5 min with different concentrations of H_2O_2 (50 μM – 80 mM). Then, the standard activity test was carried out. Each experiment was repeated two times. [20]

The results on H_2O_2 tolerance are illustrated in Figure 4 – 24. The enzyme concentration used was 250 μg mL^{-1}.

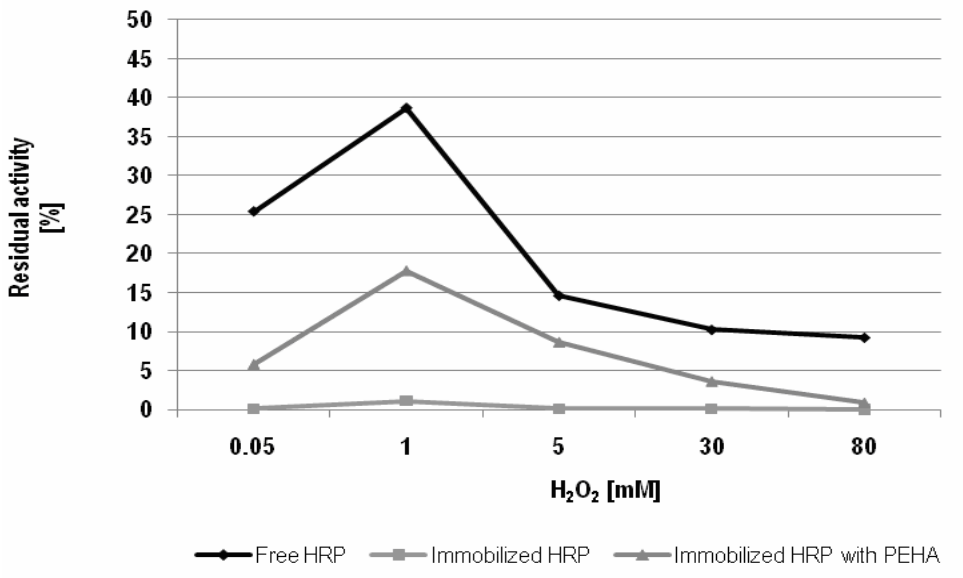


Figure 4 – 24: H_2O_2 tolerance of free HRP and bound HRP.

The soluble enzyme showed a higher H_2O_2 tolerance than the immobilized form of the same enzyme. The soluble enzyme maintained 25 – 39 % of its activity from 50 μM to 1 mM while inactivation took place when 5 mM of H_2O_2 was used. The bound HRP, co-immobilized with PEHA, was also active (6 – 18 %) at H_2O_2 concentrations between 50 μM and 1 mM. As H_2O_2 concentrations were increased from 5 mM to 80 mM, the immobilized HRP gradually lost its activity. The enzyme was 2 % active at 80 mM H_2O_2.

The immobilized HRP without the presence of PEHA, in contrast, showed minimal activity, in the whole H_2O_2 concentration range herein used. The results clearly demostrate that bound HRP, co-immobilized with PEHA, has an improved tolerance to H_2O_2. However, the free HRP has exhibited a better tolerance to higher concentrations of H_2O_2, compared to the both forms of immobilized enzyme, respectively.

5. CONCLUSIONS

Immobilization of enzymes on a prefabricated solid supports is an attractive technique to enhance the enzyme stability. Moreover, it is well documented to be relatively effective in preventing downstream contamination. In addition to that, enzyme immobilization greatly simplifies the whole bioprocess because the immobilized enzymes can be recovered and reused again in the subsequent reaction cycles, as long as the enzyme still maintains its functionality and operational stability.

Furthermore, by immobilization the enzymes may also adapt much better functional properties mediated structural rearrangement that gives them higher protection against chemical or physical agents such as inhibition by reaction products or elevated temperatures. On conclusion, supports in the form of nanostructures are becoming as the most desirable and appropriate enzyme carriers, in particular, due for their chemical inertness and size compatibility with enzymes.

In the recent years, diverse extensive studies have been dedicated to the development of highly efficient nanostructures entities for enzyme stabilization with exceptional potential application in industrially important disciplines and biological sciences, as well. Therefore, it is possible, with the help of nanotechnology, to create extremely fine and robust approaches for uses in various fields: enzyme immobilization, biocatalysis, biosensing and medically related areas.

In the present work, an easy and cheap method for preparation of chemically modified maghemite nanospheres covered with functional coatings of silica layer and AEAPTS amino-silane molecules was utilized. Furthermore, HRP was successfully immobilized and showed good catalytic activity. The optimal reaction conditions used for HRP immobilization onto magnetic nanoparticles (5 mg) were found at medium pH 4.0, 250 μg mL^{-1} of concentration of HRP and at the temperature of 25 ºC. The addition of salt, NaCl, during immobilization did not improve the binding efficiency. Moreover, higher immobilization yields were obtained when the enzyme HRP was additionally co-immobilized with the functional additive, PEHA. The optimal concentration of PEHA added was found to be at 20 mmol L^{-1}. The optimal time of co-immobilization of the enzyme HRP with PEHA amino donor was 30 min.

Nevertheless, subsequent experiments will be employed to find out the stability of HRP attached to silica coated maghemite nanoparticles, respectively.

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7. APPENDIX

7.1. Experimental measurements

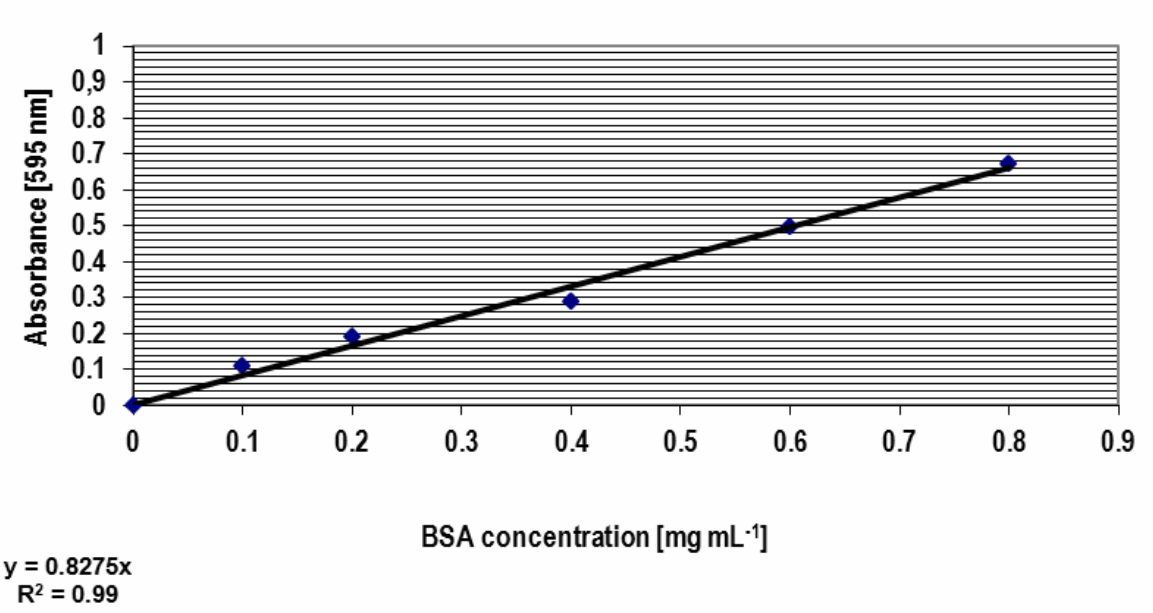
Parameter	Binding efficiency (ρ,%)						
pH	m_{NP} (mg)	$Abs_{595}(i)$ (A)	C_i (mg mL^{-1})	$Abs_{595}(f)$ (A)	C_f (mg mL^{-1})	$\Delta=c_i-c_f$ (mg mL^{-1})	ρ (%)
3	5	0.2179	0.2633	0.0897	0.1084	0.1549	58.8249
4	5	0.2159	0.2609	0.0819	0.0989	0.1620	62.0889
5	5	0.3045	0.3679	0.2903	0.3508	0.0172	4.6641
6	5	0.2891	0.3494	0.2676	0.3234	0.0260	7.4369
7	5	0.2887	0.3489	0.2708	0.3272	0.0217	6.2175
8	5	0.2613	0.3157	0.2447	0.2957	0.0200	6.3349
9	5	0.2887	0.3489	0.2768	0.3345	0.0144	4.1219
NaCl [M]							
0	5	0.2159	0.2609	0.0819	0.0989	0.1620	62.0889
0.2	5	0.1106	0.1336	0.0798	0.0964	0.0372	27.8155
0.6	5	0.1152	0.1392	0.0797	0.0963	0.0428	30.7859
1	5	0.1176	0.1421	0.0820	0.0990	0.0430	30.2850
Protein (μg mL^{-1})							
5	5	0.03605	0.0436	0.0338	0.0408	0.0027	6.2413
10	5	0.0584	0.0706	0.0503	0.0608	0.0098	13.8699
25	5	0.0877	0.1060	0.0765	0.0924	0.0135	12.7708
50	5	0.0155	0.0187	0.0000	0.0000	0.0187	100
100	5	0.0292	0.0353	0.0000	0.0000	0.0353	100
150	5	0.0359	0.0434	0.0000	0.0000	0.0434	100
200	5	0.0417	0.0503	0.0000	0.0000	0.0503	100
250	5	0.0472	0.0570	0.0180	0.0217	0.0353	61.9300
300	5	0.0535	0.0647	0.0295	0.0356	0.0290	44.8598
400	5	0.0712	0.0860	0.0556	0.0672	0.0189	21.9101
600	5	0.1009	0.1219	0.0943	0.1140	0.0080	6.5411
800	5	0.2261	0.2732	0.2220	0.2683	0.0050	1.8134
PEHA (μg)							
15 min							
50	5	0.0775	0.0936	0.0549	0.0663	0.0273	29.1156
100	5	0.0605	0.0731	0.0415	0.0502	0.0229	31.3482
150	5	0.0610	0.0737	0.0416	0.0503	0.0234	31.8033
200	5	0.0713	0.0861	0.0489	0.0590	0.0271	31.4386
30 min							
50	5	0.1143	0.1381	0.1120	0.1353	0.0028	2.0122
100	5	0.1070	0.1293	0.1014	0.1225	0.0068	5.2336
150	5	0.1117	0.1349	0.0976	0.1179	0.0170	12.5840
200	5	0.1527	0.1845	0.0620	0.0749	0.1096	59.4170
60 min							
50	5	0.0999	0.1207	0.0492	0.0594	0.0613	50.7762
100	5	0.0892	0.1077	0.0322	0.0389	0.0688	63.8811
150	5	0.0726	0.0877	0.0277	0.0335	0.0543	61.8457
200	5	0.0679	0.0821	0.0233	0.0281	0.0540	65.7585
Temperature (ºC)							
20	5	0.1213	0.1466	0.0512	0.0619	0.0847	57.7906
25	5	0.1527	0.1845	0.0620	0.0749	0.1096	59.4170
30	5	0.1213	0.1466	0.0512	0.0619	0.0847	57.7906
35	5	0.0989	0.1195	0.0659	0.0796	0.0399	33.3839
40	5	0.0777	0.0938	0.0610	0.0737	0.0201	21.4424

Parameter	Enzyme activity						
pH	$A_{immbHRP}$ (U mL^{-1})	$A_{supernHRP}$ (U mL^{-1})	$A_{washHRP}$ (U mL^{-1})	$A_{freeHRP}$ (U mL^{-1})	$A_{immbHRP}/A_{freeHRP}$ (%)	$A_{immbHRP}/A_{suprHRP}$ (%)	$A_{immbHRP}/A_{washHRP}$ (%)
3	0.6684	0.8714	0.0161	1.1135	60.0247	38.5256	1.4497
4	0.6413	1.1742	0.0474	1.0645	60.2406	37.3188	2.4406
5	0.1705	7.8516	0.6075	10.0493	1.6965	78.1308	6.0457
6	0.1351	5.5255	0.2825	7.5830	1.7819	85.5336	3.7257
7	0.1324	5.8140	0.4659	7.9498	1.6656	73.1339	4.7786
8	0.6622	7.7473	0.3830	9.8511	6.7225	80.7835	3.5780
9	1.0246	10.6489	0.1738	11.9916	8.5441	88.8032	1.4497
NaCl [M]							
0	0.6413	1.1742	0.0474	1.0645	60.2406	35.3085	4.4510
0.2	0.0487	0.5854	0.0558	4.6406	1.0487	12.6157	0.6479
0.6	0.0596	0.4594	0.0273	4.0254	1.4806	11.4127	0.6786
1	0.0185	0.3709	0.0309	4.5796	0.4050	10.3948	0.6749
Protein (µg ml^{-1})							
5	0.0587	2.1519	0.5086	63.4221	0.0925	3.3930	0.8020
10	0.0598	2.1139	0.1413	17.6173	0.3393	11.9988	0.8639
25	0.0747	2.3423	0.1493	12.7410	0.5861	18.3837	1.1721
50	0.0540	1.2750	0.1221	10.5184	0.5130	14.3122	1.1609
100	0.0663	0.5366	0.1236	4.8870	1.3572	10.9809	2.5293
150	0.0625	0.2857	0.1336	4.5414	1.3769	6.8168	2.9428
200	0.0402	0.1839	0.1575	3.4262	1.1721	5.3671	3.7797
250	0.1236	0.4899	1.2813	4.8870	2.5293	10.0247	20.8153
300	0.0000	0.2822	0.0141	9.1474	0.0001	3.0845	0.1890
400	0.3640	2.4886	0.2822	10.4510	0.0001	25.8482	2.6998
600	0.0000	0.6669	0.6669	24.7023	0.0001	3.0845	2.6998
800	0.0000	1.0736	1.0736	39.7647	0.0001	3.0845	2.6998
PEHA (µg)							
15 min							
50	0.0215	0.1230	0.1913	0.3553	6.0440	34.6154	53.8462
100	0.0534	0.0743	0.0502	0.3391	15.7534	21.9178	14.8082
150	0.2972	0.1536	0.1790	0.4129	71.9780	37.1978	33.4247
200	0.2122	0.1309	0.1159	0.2869	73.9726	46.5385	40.4110

30 min							
50	2.7558	1.7319	1.3224	3.4830	79.1209	49.7253	38.9726
100	1.0375	0.3223	0.4009	1.4305	72.5275	22.5275	28.0220
150	0.4198	0.1128	0.1034	0.5702	73.6264	19.7802	18.4932
200	0.0573	0.0175	0.0243	0.0709	80.8219	24.6575	34.2466
60 min							
50	0.0035	0.0087	0.0148	0.1580	2.1978	5.4945	9.3407
100	0.0008	0.0108	0.0147	0.1128	0.6849	8.2418	13.0137
150	0.0511	0.0216	0.0334	0.2869	17.8082	8.2418	11.6438
200	0.0769	0.0638	0.0609	0.1439	53.4247	32.6044	42.3288
Temperature (ºC)							
20	0.0006	0.0006	0.0025	0.0917	0.0847	0.0619	0.0880
25	0.0786	0.0165	0.0301	0.0883	0.1096	0.0749	0.0906
30	0.0006	0.0006	0.0025	0.0917	0.0847	0.0619	0.0880
35	0.0160	0.0027	0.0133	0.1947	0.0399	0.0796	0.0424
40	0.0026	0.0026	0.0026	0.3860	0.0201	0.0737	0.0721

H_2O_2 (mM)	**m_i (mg)**	**V (ml)**	**$A_{immbHRP}$ (U mL^{-1})**	**(%)**	**$A_{freeHRP}$ (U mL^{-1})**	**(%)**	**$A_{immbHRPPEHA}$ (U mL^{-1})**	**(%)**
0.05	5	3.5	0.0008	0.0830	0.2540	25.4011	0.0581	5.8064
1	5	3.5	0.0111	1.1053	0.3864	38.6383	0.1783	17.8330
5	5	3.5	0.0010	0.1000	0.1460	14.5957	0.0865	8.6489
30	5	3.5	0.0011	0.1128	0.1023	10.2287	0.0360	3.6011
80	5	3.5	0.0000	0.0011	0.0922	9.2245	0.0084	0.8436

7.2. Calibration curve for Bradford method.



$y = 0.8275x$
$R^2 = 0.99$

7.3. Biography.

europass

Europass Curriculum Vitae

Personal information

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E-mail(s)	taniacs_87@hotmail.com
Nationality	Spanish
Date of birth	17 September 1987
Gender	Female

Desired employment / Occupational field

Chemical engineer

Work experience

Dates	01 July 2008 - 15 August 2008
Occupation or position held	Lab auxiliary
Main activities and responsibilities	Water and food analysis
Name and address of employer	Coian S.A. 11, Bisbe Martí Ruano, 25006 Lleida (Spain)
Type of business or sector	Activities of households as employers; undifferentiated goods - and services - producing activities of households for own use

Dates	2007 - 2009
Occupation or position held	Particular teacher
Main activities and responsibilities	Teach about some subjects to younger students

Education and training

Dates	2003 - 2005
Title of qualification awarded	High School
Principal subjects / occupational skills covered	Health science, technological
Name and type of organisation providing education and training	Col·legi Maristes Montserrat de Lleida (High School) 3, Av.Catalunya, 25002 Lleida (Spain)
Level in national or international classification	4A

Dates	2005 - 2011
Title of qualification awarded	Chemical Engineering
Principal subjects / occupational skills covered	Chemistry subjects & Physical Subjects
Name and type of organisation providing education and training	UPC - ETSEIB 647, Av.Diagonal, 08028 Barcelona (Spain)
Level in national or international classification	5B

Personal skills and competences

Mother tongue(s) **Catalan / Spanish**

Other language(s)

Self-assessment	Understanding		Speaking		Writing
European level (*)	Listening	Reading	Spoken interaction	Spoken production	
English	B1 Independent user	B1 Independent user	B1 Independent user	B1 Independent user	B1 Independent user

(*) Common European Framework of Reference (CEF) level

Social skills and competences
- Team spirit gained through my work experience
- Good ability to comunicate with different people gained through my years in a student residence

Organisational skills and competences
- Responsible and polite person

Computer skills and competences
- Good command of Microsoft Office
- Good knowledge of Solidworks
- Catia basic course (summer 2010)

Artistic skills and competences
- Good sense of music gained through my ten years in a Music Conservatory, playing the piano.

Driving licence(s) B