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Cross-linking of Horseradish Peroxidase Enzyme to Fine Particles Generated by Nano Spray Dryer B-90

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RESEARCH ARTICLE

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Abstract

Enzymatic methods can be effective tools for removal of phenolic compounds from wastewater by forming reactive radicals with polymerization mechanism. Comparing with conventional chemical ways, enzymes have advantages such as low cost and high level of catalytic efficiency. At the same time, there exist some problems, e.g. the instability of enzyme structures, sensitivity to process conditions and their purification cost is high. Fine particles have been used for enzyme stabilization due to their beneficial high surface-to-volume ratio. Enzymes immobilized on fine particles may work at a broader pH, temperature and salinity range than the native enzymes. Ethyl cellulose and poly(lactic co-glycolic acid) carrier particles were prepared by nano spray drying and used for immobilizing horseradish peroxidase enzyme (HRP) by cross-linking. The optimal condition for immobilization was investigated. Fixed enzyme showed increased activity in TRIS buffer. The activity of the immobilized enzyme was enhanced at acidic and alkaline pH.

Keywords

horseradish peroxidase, immobilization, nano spray drying

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1 Introduction

Enzymes show a number of properties that make their use beneficial compared to conventional chemical ways, such as a high level of catalytic efficiency, high degree of substrate-, regio- and stereospecificity. This results in substantial process energy savings and decreased manufacturing costs [1]. At the same time, there exist some problems in the use of enzymes such as isolation and purification costs are high, the instability of enzyme structures, sensitivity to process conditions and short lifetime often need to be solved. Immobilization can be a solution which can be achieved by fixing enzymes to or within solid supports stabilizing the structure of enzymes, hence their activities. Immobilized enzymes are more resistant to environmental changes [2].

Recently, nanoparticles have been used for enzyme stabilization due to their beneficial high surface-to-volume ratio. Enzymes immobilized on fine particles may work at a broader pH, temperature and salinity range than the native enzymes [3].

Peroxidases have been reported to play an important role in wastewater treatment having the capability to oxidize and remove several organic and inorganic compounds such as phenols in the presence of hydrogen peroxide by forming reactive radicals with polymerization mechanism [4]. Horseradish *(Amoracia rusticana)* root is a commercial source for peroxidase recovery [5]. Several promising studies on detoxification of wastewater contaminated with phenols and chlorinated phenols by horseradish peroxidase enzyme (HRP) have been published [6,7].

Zhu et al. [8] investigated horseradish peroxidase activity based on inhibition of the enzyme and a decrease of the rate of coupling reaction of *p*-chlorophenol with 4-aminoantipyrine as chromogen. The peroxidase-based method was applied to the determination of ascorbic acid, norepinephrine, epinephrine, dopamine and their precursors levodopa in comparison with the official procedures. Results showed that there was no significant difference between the suggested and official method, hence the analysis using horseradish peroxidase is well applicable for pharmaceutical analysis.

Nicell and Wright [9] described a steady-state kinetic model of horseradish peroxidase activity with inhibition by hydrogen

peroxide. A colotimetric assay was used to measure the rate of color formation using phenol, 4-aminoantipyrine influenced by hydrogen peroxide concentration. They have found that HRP comparing with soybean peroxidase is catalytically more than an order of magnitude faster and less susceptible to inhibition what is beneficial for a wastewaters treatment system containing aromatic contaminants.

Qiu et al. [10] immobilized horseradish peroxidase onto nanoporous copper (NPC) surface by adsorption during incubation in aqueous solution. HRP showed enhanced thermal stability and better reusability than the free enzyme due to the multiple attachments. The electrochemical biosensor based on the immobilized HRP is suitable for the detection of *O*-phenylenediamine.

Reactive oxygen species (ROS) play important roles in the pathogenesis of various diseases. Kinetic analysis of ROS was investigated produced by phagocytes during cell based defense against bacterial and fungal infections. Quantitative measurement of ROS inside the phagosomes of living cells can be achievable with covalent attachement of ROS-sensitive dye dichlorodihydrofluorescein ($DCFH_2$) to yeast particles. Tlili et al. [11] investigated their fluorescence oxidized by horseradish peroxidase in the presence of H_2O_2.

Horseradish roots contain number of peroxidases including isoenzymes that can oxidise numerous organic and inorganic compounds by using hydrogen peroxide [12]. Horseradish peroxidase, carrying a non-covalently bound prosthetic heme group is one of the most studied peroxidases. The 44 kDa molecular weight HRP also contains two calcium binding sites. HRP has three major isoenzyme groups based on their isoelectric points: acidic (A), neutral (B and C) and basic (D and E). Among peroxidase isoenzymes, isoenzyme C is the most frequent [13].

Nano spray drying method is a commonly used technique in pharmaceutical industry producing dry powder from solutions, gels, suspensions and emulsions. Nano Spray Dryer B-90 has been chosen using piezotechnology to produce fine carrier particles. The drying gas enters in a laminar way from the top into the drying chamber and is heated up to the set temperature, then, the drying gas exits the spray dryer. The piezodriven spray nozzle generates ultra-fine droplets with narrow size distribution, which are dried to solid particles. The dried solid particles are electrostatically charged and collected at the surface of the cylindrical collecting electrode by the strong electrical field. The main advantages of the Nano Spray Dryer are the visible spraying process, short set-up times and simple cleaning; the novel electrostatic particle collector for highest yields of fine particles and only a minimal sample amount of high value product is needed to receive dry powder [14].

The aim of this work is the cross-linking of horseradish peroxidase enzyme to nano spray dried environmental friendly carrier particles such as ethyl cellulose (EC) and poly lactic co-glycolic acid (PLGA), and preserving the enzyme activity at a broader pH and temperature range. Morphology and the size of the nano spray dried particles, effect of pH and storing stability of cross-linked HRP were compared to that of free enzyme.

2 Materials and methods

2.1 Materials

Purified HRP (230 U/mg) was purchased in dry solid form from Amresco (Solon, Ohio) and stored at -20°C.). One unit of HRP activity was defined as the formation of 1.0 mg purpurogallin from pyrogallol in 20 s at 0.022 M initial pyrogallol concentration and 0.045 M H_2O_2 in 3.00 ml distilled water. Ethyl cellulose (viscosity: 4 mPa s, 5 wt% in 80:20 toluene/ethyl alcohol, 25 °C) was a kind gift from Dow Deutschland Anlagengesellschaft mbH (Germany). Poly lactic co-glycolic acid (PLGA, 50:50, Mw = 8,000, Resomer® RG 502H) containing free carboxyl end-groups was obtained from Boehringer Ingelheim, Germany. Ethyl acetate and dichloromethane (DCM) were purchased from Scharlab (Hungary). Guaiacol was purchased from Cayman Chemical Company (USA). 30% hydrogen peroxide was obtained from VWR International. N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (EDC) (~98%) and N-hydroxysuccinimide (NHS) (98%) were purchased from Sigma-Aldrich.

2.2 Spray drying with Nano Spray Dryer B-90

Ethyl cellulose and PLGA carrier particles were prepared by Nano Spray Dryer B-90 (BÜCHI Labortechnik AG, Flawil, Switzerland). The forming 1% solutions (EC was dissolved in ethyl acetate, PLGA was dissolved in DCM) were spray dried using spray caps with 7.0 μm and 5.5 μm hole sizes, which were vibrated at 60 kHz ultrasonic frequency. Nitrogen was used as drying gas; the flow rate was set to 100 L/min. The relative spray rate was set to 100%. The device was connected to a cooling unit for safety management of solvents in the closed-mode configuration with the short version of the drying chamber. The generated spray dried particles were collected from the particle collecting electrode using a particle scraping device, and then stored at room temperature for further characterization.

2.3 Immobilization of HRP

Horseradish peroxidase enzyme was covalently immobilized on nano spray dried particles, composed of EC or PLGA. 50 mg of prepared carrier was dispersed in 5 ml distilled water (DW) solution of EDC and NHS (10-10 mg, 50-50 mg or 100-100 mg), and stirred with a magnetic stirrer for 1 or 3 hours at room temperature. After the reaction time, particles were centrifuged (Heraeus Biofuge Primo R) with 8500 rpm for 10 minutes. Then, the supernatant containing the non-attached EDC and NHS was removed. After three times washing and centrifuging, 2-2 ml of HRP solution was added to the samples - containing the carbodiimide activated particles - and stirred overnight at 4°C. In other experiments, spray dried particles, EDC and NHS were stirred in 2 ml HRP solution overnight at 4°C. Then, the

non-crosslinked HRP was removed by centrifugation, and the remained particles containing cross-linked HRP were kept in a freezer for further usage.

2.4 Laser diffraction and scanning electron microscopy investigations

The particle size of the dried powders was determined by laser diffraction method (Mastersizer 2000, Malvern Instruments, UK) at room temperature. Particles were dispersed in distilled water and sonicated for 2 minutes. The average particle size was expressed in volume mean diameter.

The morphology of spray dried particles was examined by scanning electron microscopy. Particles dispersed in distilled water were dropped onto a grid and dried overnight under room temperature. Then, they were vacuum-coated for 3 minutes with a mixture of gold and palladium and examined with a Philips XL-30 Environmental Scanning Electron Microscope (ESEM) at 20kV/25kV.

2.5 Activity measurement

HRP activity was assayed using guaiacol chromophore and hydrogen peroxide (H_2O_2) as substrate while the guaiacol was peroxidated to tetraguaiacol (Nagasaka et al. 1976):

$$\text{Guaiacol} + H_2O_2 \rightarrow \text{Tetraguaiacol} + H_2O$$

In the 3.00 ml reaction mixture the final concentration of H_2O_2 was 0.001 M and the concentracion of guaiacol was 0.002 M. The assay system was carried out in a cuvette contained 0.5 ml of 0.006 M hydrogen peroxide as substrate and 1.5 ml of 0.004 M guaiacol in distilled water.

The reaction was started by the addition of 1 mg/ml dispersed particles containing cross-linked HRP. The absorbance was measured using a spectrophotometer (Pharmacia LKB-Biochrom) at 470 nm at room temperature while color change could be observed during the measurement. The absorbance was monitored after 2 minutes and the obtained initial value (absorbance at 0 s) was subtracted, when the activity values were calculated, thus avoiding the light scattering effect of the particles. All measurements were performed in triplicate.

2.6 Effect of pH

The activities of free and cross-linked HRP were measured in the case of purified HRP at pH range 4-10. The effect of pH on HRP stability was analyzed by using 0.1 M citrate (pH 4.0-6.0) and 0.02 M TRIS (pH 7.0-10.0) buffer solutions.

3 Results and discussion

3.1 Particle size distribution and morphology

Ethyl cellulose and PLGA particle sizes were determined after the spray-drying process (Fig. 1). The equivalent volume mean diameters (D [4. 3]) and d (0.1), d (0.5), d (0.9) values were obtained. The d (0.1), d (0.5), d (0.9) values mark the sizes, below which 10 %, 50 % and 90 %, respectively, of the particles are present. The majority of the particles were between 1 and 10 μm by using 7.0 μm and 5.5 μm spray caps (Table 1).

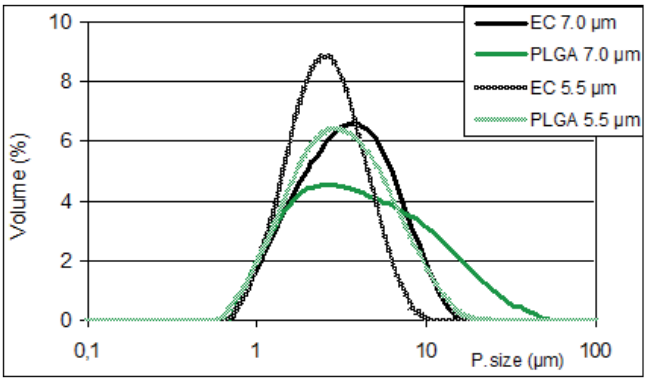

Fig. 1 Particle size distribution of nano spray dried ethyl cellulose (EC) and poly(lactic co-glycolic acid) (PLGA).

Table 1 Volume mean diameters (D [4. 3]) and d (0.1), d (0.5), d (0.9)) values of nano spray dried ethyl cellulose (EC) and poly(lactic co-glycolic acid) (PLGA) using spray cap hole size 7.0 μm and 5.5 μm.

Sample Names	D [4. 3]	d (0.1)	d (0.5)	d (0.9)
EC – 7.0 μm	4.2	1.5	3.6	8.0
PLGA – 7.0 μm	6.8	1.4	4.2	15.7
EC – 5.5 μm	3.0	1.4	2.7	5.1
PLGA – 5.5 μm	4.1	1.4	3.2	7.9

Nano spray dried EC particles showed spherical and partially doughnut-like morphology, while PLGA particles have only spherical shape using hole size 7.0 μm and 5.5 μm spray caps (Fig. 2).

By changing specific parameters or process conditions, doughnut-like particles can form related to the hydrodynamics, structural stability, and the behaviour of the droplets. Iskandar et al. [15] prepared nanostructured silica particles from a nanosize silica sol under various circumstances, and found that due to the big droplet size, high temperature, high gas flow rate and the presence of an added surfactant resulted in doughnut shape particles. The forming particle shape can be theoretically explained due to the hydrodynamic effect and the high internal surface energy that can stabilize the structure of the droplet. Particles with bigger size are not able to maintain their spherical shape during spraying, and they are deformed by losing their structural stability. Luo and Nieh [16] prepared ~1.73 μm average size doughnut shape hydroxyapatite particles by choosing the proper operating parameters such as atomization pressure or concentration during the spray drying process. Particles are able to elongate into an elliptical shape, while the surface area and therefore the surface tension increase and during a spring-back action, an inward breakdown in the elongated drops generates doughnut-shape granules due to the minimized surface-to-volume ratio.

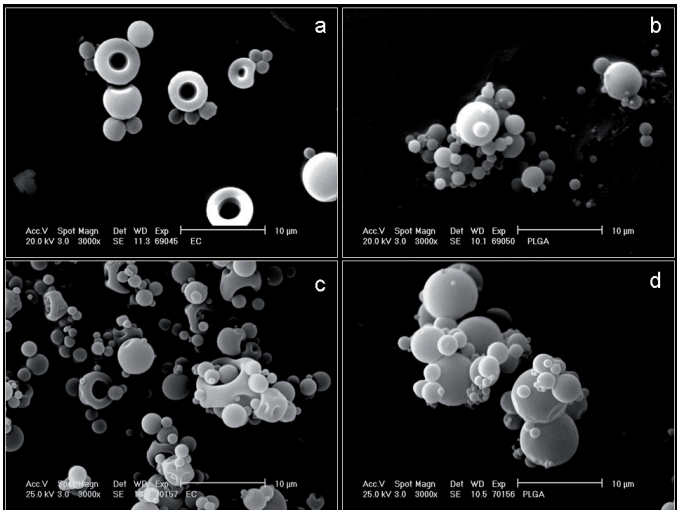

Fig. 2 SEM images of nano spray dried ethyl cellulose (EC) (a,c) and poly(lactic co-glycolic acid) (PLGA) (b,d) particles using spray cap hole size 7.0 µm (a, b) and 5.5 µm (c, d) with 3000x magnification.

3.2 HRP immobilization and activity measurement

Comparing the two types of cross-linking procedure, immobilization on previously carbodiimide activated EC particles showed better result (Fig. 3). The activity was found to be more than 5 times higher, when the supernatant did not contain the cross-linker (EDC) and its stabilizer (NHS). The reason is probably that the cross-linker can polymerize the protein, which results in its less effective attachment to the carrier support and in HRP denaturation as well.

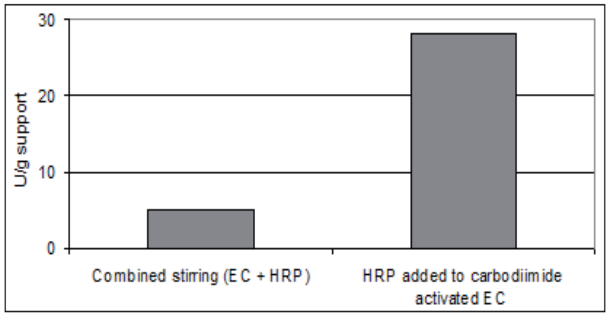

Fig. 3 Residual activity of purified horseradish peroxidase (HRP), cross-linked on nano spray dried ethyl cellulose (EC) using spray cap hole size 7.0 µm

In order to investigate the effect of reaction time, 50 mg of carrier was dispersed in 5 ml distilled water (DW) solution including EDC and NHS (10-10 mg), and stirred with a magnetic stirrer for 1 and 3 hours, respectively, at room temperature. During longer reaction time significantly higher amount of enzyme could be attached to the surface of the support (Fig. 4).

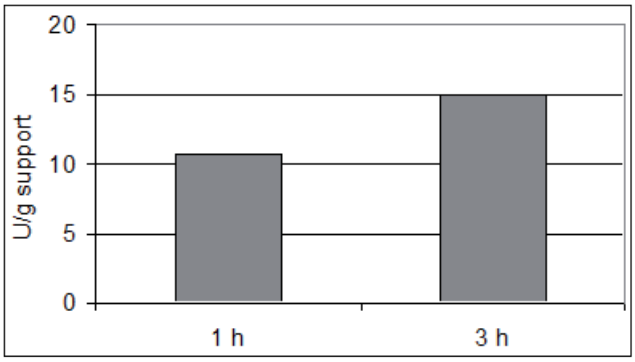

Fig. 4 Residual activity of purified horseradish peroxidase cross-linked on ethyl cellulose as a function of reaction time.

EDC and NHS quantity also influenced the attachment of HRP substantially. 10-10 mg, 50-50 mg and 100-100 mg EDC-NHS were used for analyzing the effect applying 3 hours reaction time. A maximal efficacy in cross-linking procedure was achieved by 50-50 mg EDC and NHS (Fig. 5).

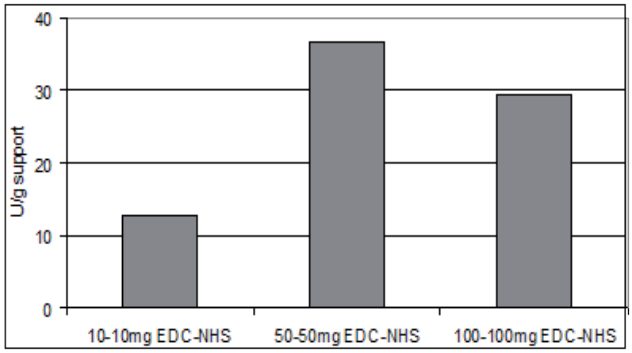

Fig. 5 Dependence of horseradish peroxidase cross-linking on ethyl cellulose particles on EDC-NHS (10, 50 and 100 mg) concentration.

Enzymes preferably work in suitable buffers, thus, the enzyme activity of HRP attached to EC and PLGA dispersed in TRIS buffer was compared to that in distilled water (Fig. 6). TRIS buffer was undoubtedly more beneficial for storing cross-linked enzyme than distilled water.

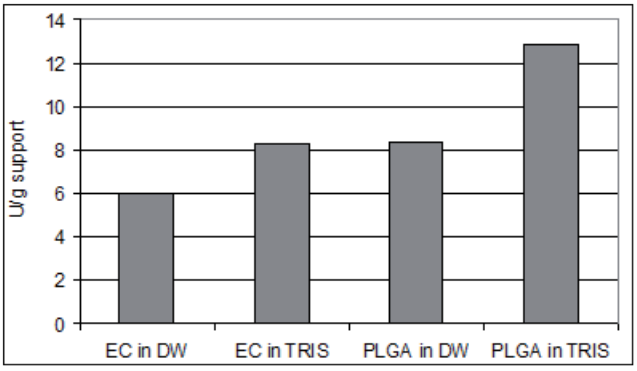

Fig. 6 Residual activity of purified horseradish peroxidase (HRP), cross-linked on ethyl cellulose (EC) and poly(lactic co-glycolic acid) (PLGA) using distilled water (DW) and tris (hydroxymethyl) aminomethane (TRIS) buffer (pH6) as dispersion medum

Higher activity can be achieved by decreasing hole size of spray caps in the nano spray drying process. More active HRP could be cross-linked on EC particles generated with 5.5 μm spray cap hole size than using hole size 7.0 μm due to the higher surface-to-volume ratio of formed support (Fig. 7).

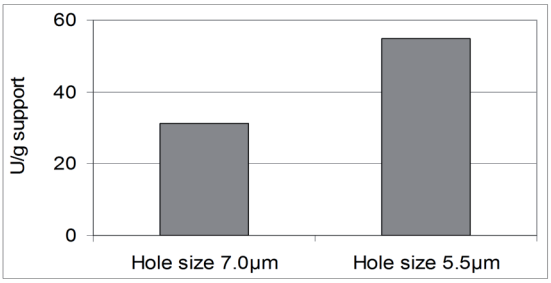

Fig. 7 Residual activity of horseradish peroxidase cross-linked on ethyl cellulose (EC), dispersed in distilled water.

3.3 pH working range

Enzymes generally operate only in a narrow pH range. If the pH moves below or over the optimal range, enzymes can stop working and denature due to conformational changes.

Working stability at the pH range of 4.0-10.0 was measured (Fig. 8). Optimum pH for both non-cross-linked enzymes was at pH 7. Immobilized enzyme activity was improved in both the acidic and the alkaline pH region by immobilization. However, it must be noted that two buffers with substantially different ion concentration were used to produce the relatively wide pH range for the investigation of pH dependence. Thus, the ionic strength of the buffers differed significantly, which can also have important effect on the activity of HRP. This influence has to be investigated in a more detailed study.

Other authors also faced with the increasing pH working range of HRP after immobization [17]. Lai and Lin [5] immobilized HRP on porous aminopropyl glass beads for the removal of *p*-chlorophenol. The pH working range was shifted downward by one pH unit from 8.0–9.0 to 7.0–9.0 while during the removal of *p*-chlorophenol, pH range was also broadened toward acidic range with one pH unit from 7.5–9.5 to 6.5–9.5. Torabi et al. [18] covalently immobilized HRP on perlite surface activated with 3-aminopropyltriethoxysilane via glutaraldehyde. Immobilization shifted optimum pH of enzymes to the lower pH. The optimum pH of the free enzyme is at around 7 while pH optimum of immobilized HRP shifted slightly to the lower pH, but significant increasing could be observed in the activity in the case of immobilized HRP in acidic condition.

4 Conclusions

Spherical and doughnut shape ethyl cellulose and spherical poly (lactic co-glycolic acid) carrier particles were generated by Nano Spray Dryer B-90. Smaller spray cap hole size resulted in higher activity of fixed enzyme due to the increasing surface-to-volume ratio of the produced particles. The immobilization

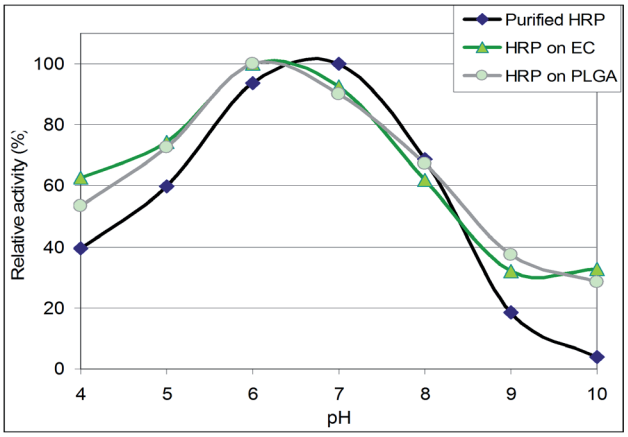

Fig. 8 pH stability of the free and cross-linked horseradish peroxidase (HRP) immobilized on ethyl cellulose (EC) and poly(lactic co-glycolic acid) (PLGA)

of horseradish peroxidase to these solid supports was achieved by fixing HRP with EDC cross-linker. The optimal condition for immobilization was found using 50 mg of EDC cross-linker and 50 mg NHS additive applying 3 h reaction time. Fixed enzyme showed increased activity in TRIS buffer. The activity of the immobilized enzyme was enhanced at both acidic and alkaline pH.

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