Immobilization of chemically modified horseradish peroxidase within activated alginate beads

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Abstract
Immobilization of horseradish peroxidase (HRP) within alginate beads was enabled by chemical modification of the enzyme and polysaccharide chains. HRP and alginate were oxidized by periodate and subsequently modified with ethylenediamine. Highest specific activity of 0.43 U/ml of gel and 81% of bound enzyme activity was obtained using aminated HRP and alginate oxidized by periodate. Immobilized enzyme retained 75% of its original activity after 2 days of incubation in 80% (v/v) dioxane and had increased activity in basic solutions compared to native enzyme. During repeated use in batch reactor for pyrogallol oxidation immobilized peroxidase retained 75% of its original activity.

Keywords: periodate, ethylene diamine, peroxidase, immobilized.

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Horseradish peroxidase (HRP) is one of the most studied peroxidases from plants that can be used for organic synthesis of chemicals like DOPA and bisphenols [1], for removal of polutants such as phenol and aniline from wastewaters [2] and for manufacturing of biosensors [3].

In order to be used in industry, it is necessary to decrease the cost of the enzyme. This can be achieved by repeated use of the same enzyme batch for prolonged period of time. In order to do so, it is necessary to develop efficient method for enzyme immobilization that will facilitate easy removal of the catalyst from reaction mixture without leaking or losing activity [4]. Immobilization within alginate is one of the most common methods for enzyme and cell immobilization since it does not require expensive chemicals or complicated setup [5]. Since alginate macromolecular gel has large pore diameter, low molecular weight enzymes like peroxidases, can easily diffuse out [6]. In order to overcome this problem several approaches were tried including coating with polylysine or chitosan [7]. Chemical modification of polymers or enzymes can introduce new functional groups that also promote enzyme immobilization and adsorption to the gel [8].

Alginate was used previously for immobilization of soybean peroxidase [9], turnip peroxidase [10] and horseradish peroxidase [6,11] but in most cases, due to low molecular weight, approximately 45 kDa, leakage of the peroxidase from alginate gel was significant.

In the presented research article we developed a novel method for peroxidase immobilization within alginate hydrogel by chemically modifying enzyme molecules and introducing new functional groups into alginate through periodate oxidation.

MATERIALS AND METHODS

Immobilization of native HRP within native alginate

Sodium alginate was dissolved in the reagent grade water for preparing a 2% (w/v) solution. The gel was stored at 4 °C till further use. Solution of enzyme in water (concentration 1 mg/ml) was added to alginate gel to yield final concentration of 0.01 mg/ml. To perform matrix beading, a needle syringe was filled with the solution and the beads were obtained by dripping the bead-forming solution into a solution of $CaCl_2$ (5.5%) and mildly stirring for 1 h to allow beads hardening. The beads were then washed with the same solution of $CaCl_2$ (20 ml) and stored in HEPES buffer (10 mM, pH 7) with 5mM $CaCl_2$ at 4 °C.

Activation of alginate by oxidation

Sodium alginate (1 g) was added to 100 ml of sodium periodate solution (50 mM) at 4 °C in the dark for activation. The reaction was stopped after 24 h by adding an equimolar amount of glycerol. NaCl (2 g) was then added to the mixture, in order to facilitate the subsequent precipitation in the excess of ethanol (3 volumes). The precipitate was collected, redissolved in distilled water (10 ml) and dialyzed for 24 h to remove completely the sodium periodate and other chemicals

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or byproducts. After that, the polymer was precipitated and washed three times in 100% (v/v) ethanol, filtered, then dried with pure acetone. The obtained activated alginate product was conserved at room temperature.

Immobilization of native HRP within periodate oxidized alginate

Activated alginate was dissolved in 0.2 M $NaHCO_3$, pH 8.0, to yield final concentration of 2% (w/v), while stirring. After dissolving, HRP was added to the alginate solution, final concentration 0.01 mg/ml. Enzyme immobilization was continued for 24 h at 4 °C. Finally, 50 mM Tris buffer pH 7.0 (up to 1/4 of the final volume), was introduced drop by drop into the solution to quench the remaining noncoupled (if any) carbonyl groups. The bead forming solution was obtained by addition of 2% (w/v) of native alginate at 1:1 ratio to the alginate-HRP conjugate solution. Additional non-modified alginate is essential to improve the mechanical properties of beads, because the structure of activated alginate was altered during periodate oxidation. The same procedure was employed for alginate matrix beading as described previously for native alginate.

Modification of HRP with etylenediamine

Peroxidase (1 mg) was dissolved in 2 ml of 50 mM $NaHCO_3$ buffer, pH 8.0, followed by adding 200 μL of 50 mM sodium periodate. The solution was left at 4 °C in the dark for 6 h, stirring occasionally. After 6 h, 10 μL of glycerol was added to the final solution and allowed to settle for 30 min. Finally, the solution was placed for dialysis against distilled water.

Periodate oxidized HRP was mixed with ethylenediamine (50 mM final concentration, pH 8.0) for 2 h, followed by addition of $NaBH_4$ in 0.1 M NaOH,final concentration being 1 mg/ml, for 60 min at 4 °C. Unreacted ethylenediamine was removed by dialysis against 0.1 M sodium phosphate buffer, pH 7.0, and after that, against distilled water. Obtained HRP was kept in a freezer.

Immobilization of aminated HRP in periodate oxidized alginate was performed as previously described for immobilization of native HRP within periodate oxidized alginate.

Modification of periodate oxidized alginate with ethylenediamine

Periodate oxidized alginate (0.4 g) was dissolved in 10 ml of $NaHCO_3$ buffer (pH 8.0), then 10 ml of 0.1 M ethylenediamine was added and the mixture was left in the dark for 30 min. After that 0.05 g $NaBH_3$ (CN) was added and left overnight. This solution was placed for dialysis against distilled water.

Immobilization of periodate oxidized HRP in aminated alginate was performed as previously described for immobilization of native HRP within periodate oxidized alginate.

Enzyme activity

Pyrogallol solution (13 mM) in 10 mM HEPES buffer (pH 7.0) was prepared. Enzyme immobilized beads (50–80 mg) were added to 3 ml of thus prepared solution along with 30 μl of 0.97 M H_2O_2. The mixture was kept for constant stirring on magnetic stirrer and aliquots were taken at 0, 5, 10 and 15 min post-incubation. Absorbance was measured at 420 nm.

Unbound activity was measured in a solution of $CaCl_2$ after gel beading. "Leakage" was measured after 24, 48 h and 5 days, and retained specific activity was measured after 5 days.

RESULTS AND DISCUSSION

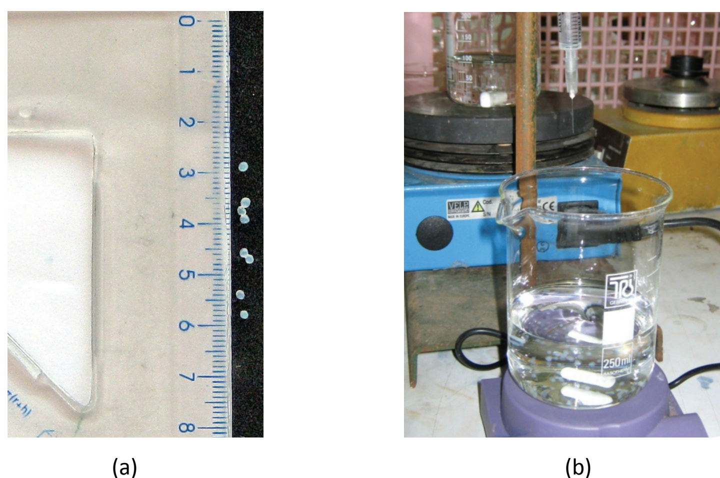
Immobilization of HRP

In order to efficiently immobilize HRP within alginate beads, different methods of enzyme and alginate modification were tried. Three types of alginate were used: native one, alginate oxidized by periodate and alginate modified with ethylene diamine after periodate oxidation. Three types of HRP were also used, native enzyme, enzyme oxidized by periodate and enzyme modified with ethylene diamine after periodate oxidation. Enzyme concentration used for immobilization was fixed at 0.01 mg/mL, alginate concentration was 2% (w/v) and in order to have better mechanical properties, modified alginate was mixed in 1:1 ratio with 2% (w/v) native form, just before immobilization. Ionotropic gelation with $CaCl_2$ gave beads with average diameter of 1.5 mm (Figure 1).

Four different combinations of native and modified enzyme and alginate were tried. First, native HRP was immobilized within native alginate and in the second experiment within periodate oxidized alginate. Third, periodate oxidized HRP, according to the protocol we previously developed [12] in order to introduce aldehyde groups, was immobilizied within alginate modified with ethylenediamine after periodate oxidation. Forth experimental setup was HRP modified with ethylenediamine after periodate oxidation immobilized within periodate oxidized alginate.

Based on analysis of enzyme activity after immobilization, it can be concluded that modified alginate and modified enzyme exhibited higher amount of activity compared to the classical method for HRP immobilization within alginate beads (Table 1).

Highest activity of 0.43 U/g was obtained by encapsulation of aminated HRP within oxidized alginate. The same conjugate showed also highest retention of activity after 5 days of washing, which is indicative that enzyme was not only entrapped within alginate matrix, but also covalently bound via aldehyde groups present in oxidized alginate. Aminated HRP immobilized within oxidized alginate was further characterized with res-



(a) (b)

Figure 1. Beads with immobilized enzyme after gelation in $CaCl_2$ solution (a) and setup used for bead formation (b).

pect to pH optimum, stability in organic solvent and kinetic properties.

pH Influence on activity

pH Optimum was determined using acetate, HEPES and glycine buffer. Compared to native HRP, aminated form immobilized within oxidized alginate showed pH optimum shift towards low values (pH < 7), as in Figure 2.

While in previous studies of peroxidase immobilization within nonmodified alginate, pH optimum was not changed [11], in our case change in pH optimum was probably result of introduced charged groups

Table 1. Specific activity, bound activity, percentage of binding, immobilization yield and residual activity within alginate beads for different immobilization methods. Percentage of binding is defined as a ratio of bound activity and activity that was added per gram of alginate gel during immobilization. Immobilization yield is defined as a ratio of specific and bound activity. Residual activity is defined as a ratio of specific activity immediately after immobilization and specific activity after 5 days of rinsing in buffer

Immobilization method	Specific activity, U/g	Bound activity, U/g	Percentage of binding, %	Immobilization yield, %	Residual activity, U/g	Residual activity, %
Alginate + HRP	0.15	0.80	9.69	18.4	0.063	42.6
Oxidized alginate + HRP	0.13	1.11	9.69	11.7	0.081	62.3
Aminated alginate + oxidized HRP	0.15	1.73	14.1	8.92	0.074	48.0
Oxidized alginate + aminated HRP	0.43	5.76	81.4	7.43	0.409	95.6

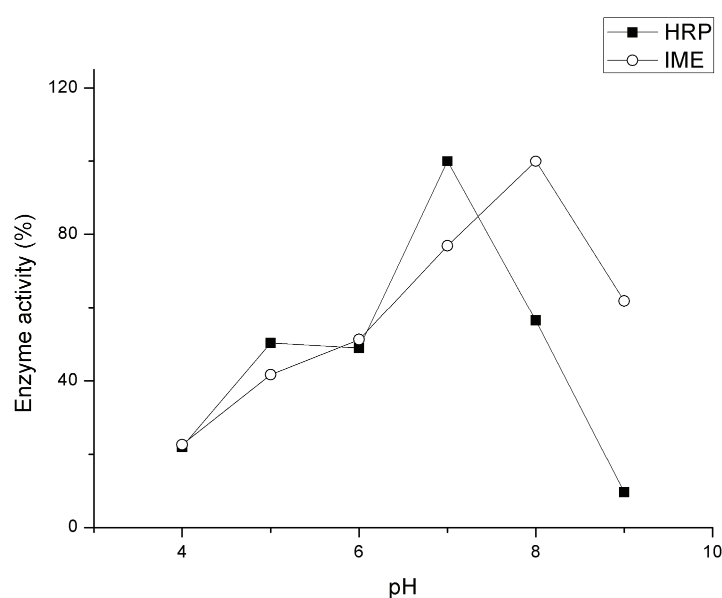


Figure 2. Influence of pH on activity of free (filled square) and immobilized HRP (open circle).

within alginate due to chemical modification. Wider pH optimum and higher activity at more basic pH values of immobilized HRP makes it more suitable for industrial application since it is less sensitive to changes in pH.

Stability in the presence of dioxane

Since HRP is also used for polymer synthesis in organic solvents, stability in these media is important for its application. Stability of our immobilized HRP was measured in 80% (v/v) dioxane at room temperature (25 °C). After incubation in dioxane water mixture, immobilized enzyme was washed with buffer and residual activity was measured in water solution (Figure 3).

Stability of immobilized enzyme in the presence of dioxane increased substantially. While free enzyme was losing 90% of its original activity after 1 day of incubation, immobilized enzyme retained more than 75% of the original activity after 2 days of incubation in 80% (v/v) dioxane.

Determination of K_m and V_{max}

K_m value for pyrogallol of free enzyme is 2.24 mM, while our immobilized enzyme showed K_m value of 4.86 mM (Figure 4).

These results were in good agreement with previous reports for enzymes immobilized within alginate gels and they appear to be a result of diffusional limitations that occur within alginate beads.

Repeated cycle testing in batch reactor

At the end aminated HRP immobilized within beads made from oxidized alginate was tested in a batch reactor for repeated use during pyrogallol oxidation.

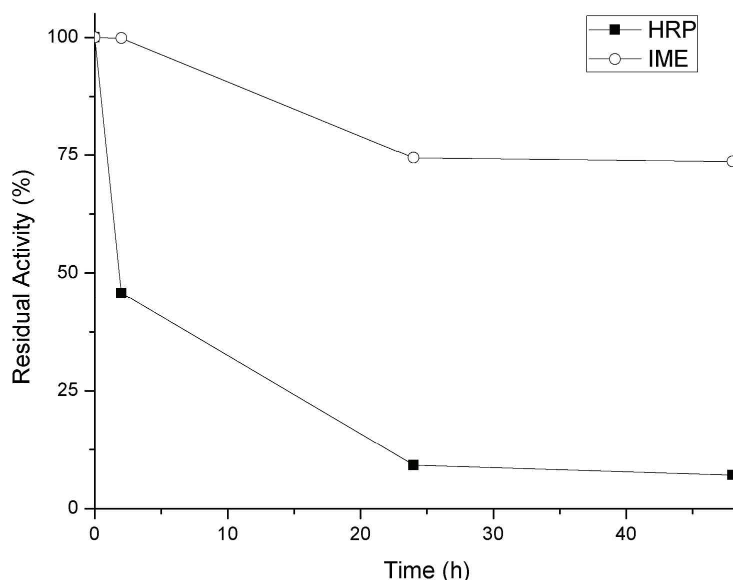


Figure 3. Influence of incubation time in 80% (v/v) dioxane on residual activity of free and immobilized HRP.

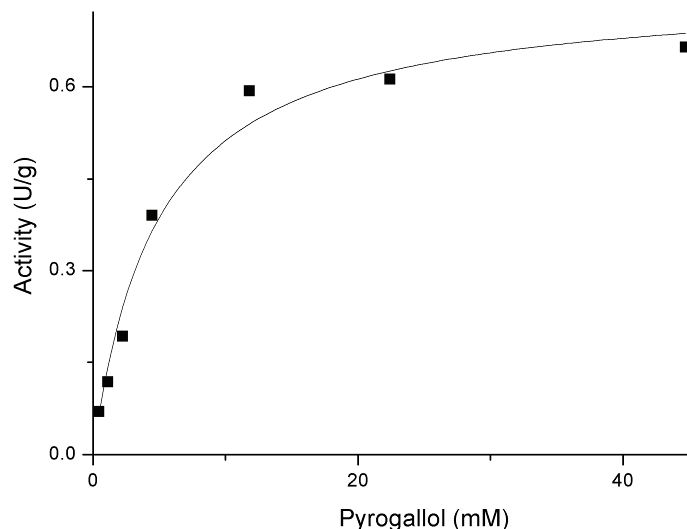


Figure 4. Michaelis–Menten curve for immobilized HRP.

Each cycle lasted for 6 h and afterwards, amount of oxidized pyrogallol was determined spectrophotometrically while beads were washed and reused (Figure 5).

After first cycle, immobilized enzyme lost some of the activity, but later activity remained unchanged through 5 cycles of repeated use. This was a better result than previously reported for HRP immobilized within native alginate [6] where activity after 5 cycles dropped below 40 % of the original value. This makes our immobilized HRP a suitable candidate for application in industry where operational stability is of the utmost importance.

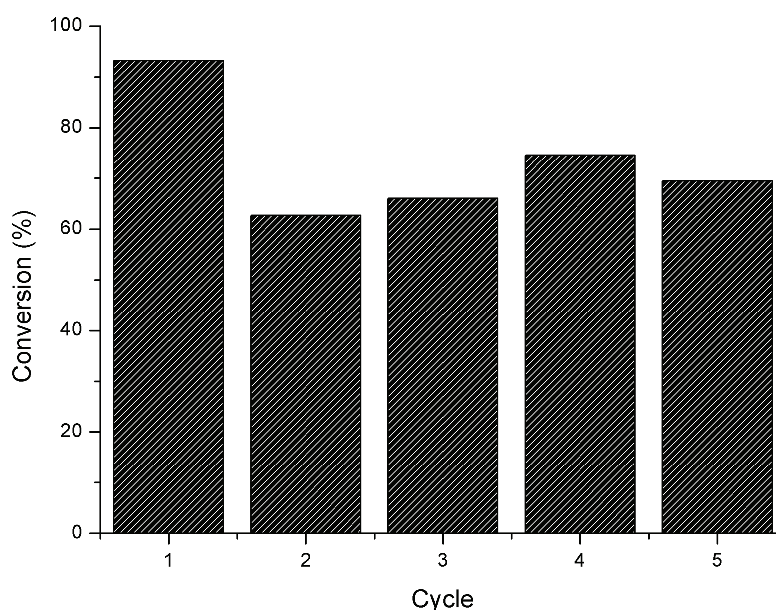


Figure 5. Activity of immobilized HRP during repeated batch run.

CONCLUSION

We have developed the protocol for efficient immobilization of HRP within alginate beads. In order to improve immobilization efficiency, HRP was modified with ethylenediamine and at the same time alginate was oxidized with sodium periodate to introduce reactive aldehyde groups into polysaccharide backbone. Due to those modifications enzyme was covalently bound to the alginate beads and could not be released after subsequent washing or repeated use in batch reactor. Immobilized enzyme also showed high stability in the presence of organic solvents and increased tolerance to pH changes. In a batch reactor it could be used in 5 consecutive rounds for pyrogallol oxidation without significant loss of enzymatic activity. Therefore, thus prepared immobilized HRP is a suitable candidate for industrial application in phenol removal or polymer synthesis in monophasic organic solvent and aqueous solutions.

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IZVOD

IMOBILIZACIJA HEMIJSKI MODIFIKOVANE PEROKSIDAZE IZ RENA UNUTAR AKTIVIRANIH ALGINATNIH KUGLICA

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(Naučni rad)

Imobilizacija peroksidaze iz rena unutar alginatnih kuglica je poboljšana hemijskom modifikacijom enzima i polisaharidnih lanaca. Peroksidaza i alginat su oksidovani perjodatom i naknadno modifikovani etilendiaminom. Najveća specifična aktivnost od 0,43 U/ml gela i 81% vezane aktivnosti je dobijeno korišćenjem aminovane peroksidaze i alginata oksidovanog perjodatom. Imobilizovani enzim je zadržao 75% originalne aktivnosti nakon 2 dana inkubacije u 80% (v/v) dioksanu i imao je povećanu aktivnost pri baznim pH vrednostima u poređenju sa nativnim enzimom. Tokom višestruke upotrebe u šaržnom reaktoru za oksidaciju pirogalola imobilizovana peroksidaza je zadržala 75% početne aktivnosti.

Ključne reči: Perjodat • Etilendiamin • Peroksidaza • Imobilizovan