Cent. Eur. J. Chem. • 7(3) • 2009 • 423-428
DOI: 10.2478/s11532-009-0041-z

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Central European Journal of **Chemistry**

Stabilization of horseradish peroxidase by covalent conjugation with dextran aldehyde against temperature and pH changes

Research Article

Melda Altikatoglu[1*], Candan Arioz[2], Yeliz Basaran[2], Huriye Kuzu[2]

[1]*Faculty of Arts and Sciences, Department of Chemistry, Yildiz Technical University, Davutpasa Campus, 34210 Esenler, Istanbul, Turkey*

[2]*Faculty of Chemical and Metallurgical Engineering, Department of Bioengineering, Yildiz Technical University, Davutpasa Campus, 34210 Esenler, Istanbul, Turkey*

Received 30 September 2008; Accepted 29 December 2008

Abstract: Stabilization of Horseradish Peroxidase (HRP; EC 1.11.1.7) against temperature and pH via the formation of the conjugates obtained by multipoint covalent bonding of dextran aldehyde (DA) to the enzyme were studied. Hence, three different molar weighted dextrans (17.5 kD, 75 kD, 188 kD) were covalently bonded to purified enzyme with different molar ratios (n_{HRP}/n_{DA} 20/1, 10/1, 1/1, 1/5, 1/10, 1/15, 1/20). The thermal stabilities of the obtained conjugates were evaluated with the activities determined at different temperatures (25, 30, 35, 40, 50, 60, 70, 80°C) applying 60 minutes incubation time. Conjugates formed were characterized by gel-permeation chromatography (GPC) and fluorescence techniques. The conjugate synthesized using dextran 75 kDa with n_{HRP}/n_{DA} 1/10 molar ratio showed better thermal stability than other conjugates and purified enzyme at pH 7. This conjugate also has wider activity pH range than purified enzyme. In addition, mentioned conjugate at pH 7 had very long storage lifetime compared to purified enzyme at +4°C and room temperature; which is considered a favorable feature for usage in practice.

Keywords: *Enzyme stabilization • Covalent conjugate • Horseradish peroxidase • Dextran • GPC*

1. Introduction

Thermostable enzymes present many advantages for several applications, such as detergent manufacturing, food and starch processing, production of fructose corn syrup *etc*. [1-4]. Most enzymes are not stable at high temperatures. This feature limits their usage and storage under such conditions [5-8]. To develop stable enzymes using protein engineering, immobilization techniques, stabilizing additives and chemical modification offers opportunities for practical applications [9-11]. Horseradish peroxidase is used as a reagent for organic synthesis, biotransformation, as well, as in coupled enzyme assays, chemiluminescent assays, immunoassays and the treatment of waste waters. Improvements to desirable qualities of the enzyme such as its relatively good stability in aqueous and nonaqueous solvent systems are actively sought as an outcome of chemical modification, site-directed mutagenesis and directed evolution studies [12,13]. Some authors suggest that the modification of the enzyme surface with a hydrophilic polymer could be a good strategy to form a shell around the enzyme molecule [14-18]. The chosen polymer should be reactive towards the groups located on the enzyme surface [9,19-24]. Dextran is a flexible polymer that accomplishes these requirements and its aldehyde derivative has been used to crosslink multimeric enzymes generating hydrophilic environments [25-28].

Horseradish peroxidase (HRP; EC 1.11.1.7), a heme-containing enzyme that utilizes hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds, notably used in the diagnostic industry and has great commercial importance [2,29,30]. HRP is a glycoprotein having approximately 18% N-linked oligosaccharides in its composition. The function of two Ca^{2+} ions in HRP molecule is to maintain enzyme

* *E-mail: maltikatoglu@yahoo.com*

conformation. Fifteen HRP isoenzymes have been isolated and are generally referred as codes based on their isoelectric points [5,31,32].
Peroxidases are enzymes whose primary function is catalyzing the oxidation of variety of substrates, AH_2, by peroxides. It has three different forms known as Compound I, II and III [12,33].

Resting Form of Enzyme + H_2O_2 ⟹ Compound I + H_2O
Compound I + AH_2 ⟹ Compound II + $AH^{\cdot}$
Compound II + AH_2 ⟹
⟹ Resting Form of Enzyme + $AH^{\cdot}$ + H_2O

Intracellular stability of HRP-Dextran conjugate have been reported by Mumtaz and Bachhavat [34]. However, *in vitro* stability has not been presented in the literature. In this study, we aimed to obtain *in vitro* the stable horseradish peroxidase-dextran aldehyde conjugates at various temperatures and pH levels for the first time. Therefore, horseradish peroxidase covalently bonded to three different molecular weighted dextrans with different molar ratios. The stabilities of the conjugates towards temperature and pH were evaluated with the activities determined at different temperatures using a one hour incubation time.

2. Experimental Procedures

2.1. Materials

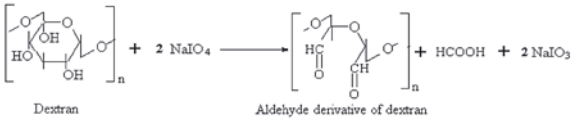
Horseradish Peroxidase (E.C. 1.11.1.7) (Mw ~ 40.000 Da) and o-dianisidine were purchased from Fluka. Concanavalin A-Sepharose 4B column material and dextrans from *Leuconostoc mesenteroides* with different molecular weights (M_w 17.500 Da, M_w 75.000 Da, M_w 188.000 Da, were obtained from Sigma Chemical Co. (St. Louis, MO). Regenerated Cellulose membrane was purchased from Millipore (Dia 25 mm, NMWL 10.000). All other chemicals used were analytical grade.

2.2. Enzyme purification procedure

The protein content of the purchased HRP (Fluka) was determined as 50% (A_{280} / 1.34). GPC (Viscotek GPCmax VE2001 GPC Solvent/sample module) chromatograms showed that there were impurities in this type of commercial enzymes. In order to synthesize the conjugates, it was decided that using purified enzyme would be better for this study. Purchased HRP was purified by Affinity Chromatography using Concanavalin A-Sepharose 4B (con A-Sepharose 4B) as column material. In order to remove all the unbound impurities, the column was washed with 0.1 M acetate buffer, pH 6 containing 0.1 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. To elute the enzyme which was bound to the column material, the column was washed with 0.1 M acetate buffer, pH 6 containing 0.1 M methyl-α-D-mannopyranoside and the fractions were collected [35]. Absorbances at 280 and 403 nm were measured with UV spectrophotometer and the Rein Heitszahl (RZ) values (A_{460} / A_{280}) for purified and commercial enzymes were calculated. The RZ value for purified HRP was 2.1 whereas RZ value of the purchased enzyme was only 0.85. Purified enzyme was concentrated in ultrafiltration cell with Regenerated Cellulose membrane (Dia 25 mm, Mw 10.000) by washing 2 times with distilled water and 2 times with 0.01M phosphate buffer, pH 7. RZ value of the enzyme was determined as 2.24.

2.3. Preparation of dextran aldehyde derivatives

Freshly prepared $NaIO_4$ solution (8 g dissolved in 70 mL distilled water) was added slowly over dextran solution (3.33 g dissolved in 30 mL distilled water) and kept stirred in darkness for 24 hours at room temperature. The final solution was dialysed against distilled water for 24 hours and aldehyde derivative of dextran was recovered by freeze drying [9]. The reaction scheme is given below.



Scheme 1. Formation of aldehyde derivative of dextran.

2.4. Preparation of HRP-Dextran aldehyde conjugates

For synthesizing enzyme-polymer conjugates, an appropriate amount of dextran aldehyde derivative was calculated by making enzyme amount (0.1 mg mL^{-1}) constant according to the formula given below:

$$\frac{n_{HRP}}{n_{DA}} = \frac{C_{HRP} \cdot M_{DA}}{C_{DA} \cdot M_{HRP}} = 1/1; 1/5; 1/10; 1/15; 1/20; 10/1; 20/1$$

c - concentration (mg mL^{-1}); M - molar weight
Calculated amounts of dextran aldehyde for the mentioned ratios were dissolved in phosphate buffer, pH 7 as well as purified enzyme. The reaction was initiated by mixing enzyme (2 mL) and dextran aldehyde (2 mL) solutions together and incubation continued for 16 hours at 25°C. As a result, a schiff base was formed between aldehyde groups of dextran derivative and amine groups of enzyme. Afterwards 5.6 mL of cold (+4°C) 100 mM sodium bicarbonate pH 8.5 was added

in order to increase pH of reaction media. To reduce the schiff base ocurred and unreacted aldehyde groups of dextran derivative, 9.6 mg of sodium borohydride was added. This solution was stirred for 15 minutes and then 9.6 mg of sodium borohydride were then again added. Reduction was continued at +4°C for 15 minutes and finally the solution pH was adjusted to 7 [9].

2.5. Activity determination

The test tubes containing 885 µl phosphate buffer were incubated in stirred waterbaths at working temperatures in order to adjust the temperatures. The reaction was started by adding 20 µL o-dianisidine, 10 µL enzyme solution and finally 10 µL hydrogen peroxide as the initiator to the test tube respectively. After 10 minutes, the reaction was stopped by adding 75 µL 1 M NaOH to reaction media and A_{460} values were recorded. For repeatibility of activity measurements, five consecutive applications were carried out for each data point in same condition and recorded absorbance values are averaged. Total activity in units was calculated with these absorbance values.

$$U/mg = \frac{A460 \times 106}{\varepsilon \times t \times cHRP}$$

ε - molar absorption coefficient of o-dianisidine (11.300)
t - incubation time (min)
C_{HRP} - HRP concentration (mg mL^{-1})
A_{460} - Absorbance at 460 nm

Thermal stabilities of the synthesized conjugates, purified HRP and commercial enzyme were evaluated with the activities determined according to the procedure described above. But in this procedure enzyme/conjugate solutions were kept at different working temperatures for 60 minutes in waterbaths and activity determination was performed later at pH 7. Meanwhile the stability of purified enzyme and conjugate with ratio n_{HRP}/n_{DA} 1/10 was also determined by repeating the same procedures for each pH.

2.6. Characterization of the conjugate with GPC (Gel-Permeation Chromatography) and fluorescence techniques

Molecular weight distributions of dextran aldehyde, purified enzyme and conjugate with the ratio n_{HRP}/n_{DA} 1/10 were determined by gel-permeation chromatography (Viscotek GPCmax VE2001 GPC Solvent/sample module) on a 7.9 mm × 50 cm Shim-Pack Diol 300 column with UV and RI (refractive index) detectors. The fractions were eluted at 1 mL min^{-1} with 0.1 M PBS buffer (pH 7) containing 0.15 M NaCl and 7.5 mM NaN_3.

Fluorescence measurements for the enzyme and mentioned conjugate were carried out using a PTI QM-4 Steady State spectrofluorophotometer. The excitation (Ex.) and emission (Em.) slit widths were set to 2 nm. Sample excitation was performed at 297 nm, while the emission spectrum was recorded in the range 310 – 500 nm. The working temperature of the cell was maintained at 60°C by circulating water through the thermostatted cuvette holder.

3. Results and Discussion

After the occurrence of covalent bonding between aldehyde groups of dextran derivative and primer amino groups of enzyme, the peak of aldehydes observed at about 2939 cm^{-1} using the Fourier Transform Infrared (FTIR) disappeared.

In order to determine the formation and molecular weight distribution of conjugates, gel-permeation chromatography (GPC) with UV and RI (refractive index) detectors were used [21,36,37]. A broad, short peak of conjugate in front of the narrow enzyme peak on the chromatograms (Fig. 1a and b) relates to the formation of conjugate (n_{HRP}/n_{DA} 1/10) with the appearance of higher molar weight compared to enzyme and dextran (all results were not submitted). Chromatograms also reflects the molecular weight distributions of the samples.

The intrinsic fluorescence of HRP is highly dependent on the fluorescence energy transfer from one tryptophan (Trp 117) to heme. Hence, changes in the structure of the heme cavity affecting the distance and orientation between the heme and tryptophan, affects the intrinsic fluorescence of the enzyme, because of the variation in the microenvironment surrounding the trypthophan residue [31,38]. The fluorescence intensities of HRP and HRP-dextran conjugate (n_{HRP}/n_{DA} 1/10) depending on time at 60°C were (Ex 297 nm, Em 330 nm) given at Fig. 2.

The intensity of tryptophan fluorescence of pure HRP increased and reached a maximum at the sixth minute with incubation at 60°C, because of the increase in distance between heme and tryptophan or a change in the relative orientation. After that point, the change in fluorescence intensity of enzyme was observed to decrease. The fluorescence intensity of conjugate was not changed significantly for 30 minutes at this temperature. Hence the fluorescence results implied that heating at 60°C caused a change in conformation of pure enzyme; it did not affect HRP-dextran aldehyde conjugate conformation which implies its stability.

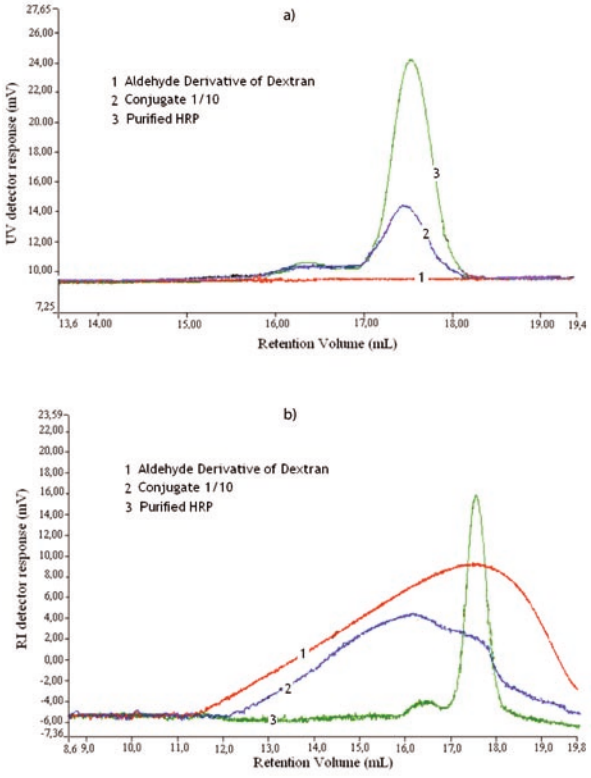


Figure 1. Chromatograms of HRP-dextran aldehyde conjugate (n_{HRP}/n_{DA} 1/10) and purified HRP; a) with UV detector; b)with RI detector.

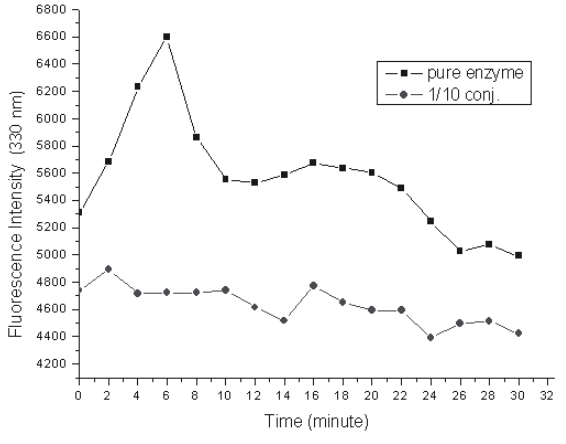


Figure 2. Fluorescence intensities of conjugate (n_{HRP}/n_{DA} 1/10) and purified HRP depending on time (Ex. 297 nm, Em. 330 nm) at 60°C, pH 7.

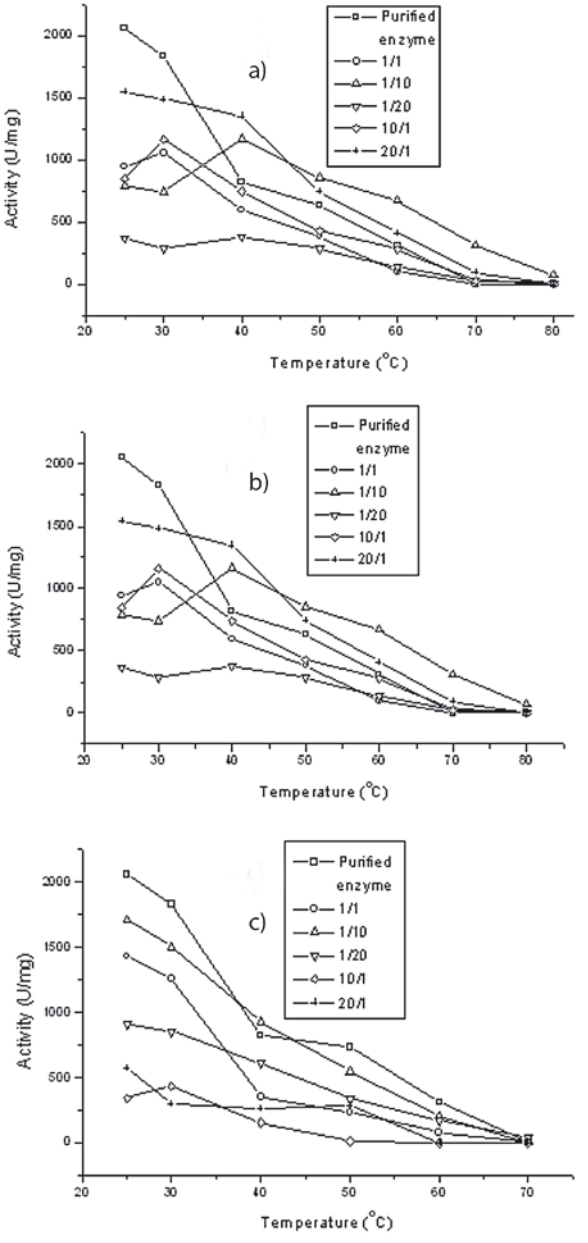


Figure 3. Thermal stabilities of HRP-dextran aldehyde conjugates with different molar ratios (n_{HRP}/n_{DA} 20, 10, 1, 1/10, 1/20) obtained using three different molar weighted dextrans at pH 7: a) with dextran 17 500 Da; b) with dextran 75 000 Da; c) with dextran 188 000 Da; activity determination with 60 minutes incubation at working temperature.

As shown in Fig. 3a, b and c, activity decline of purified enzyme was very fast with increase in temperature. Conjugates had however a higher resistance *versus* temperature. High resistance against temperature may be because of the formation of a thick polymer cover around the enzyme molecules. Possibly because of the loosing of formed thick dextran cover, activities of conjugates with 75 kDa dextran increased from 25°C to 40°C, and decreased slowly from 40°C to 80°C. Conjugates synthesized using 75 kDa dextran only had activities at 80°C. And also, only the conjugate n_{HRP}/n_{DA} 1/10 with 75 kDa dextran, showed higher activity than other conjugates and enzyme over 40°C when 60 minutes incubation was applied. Conjugates

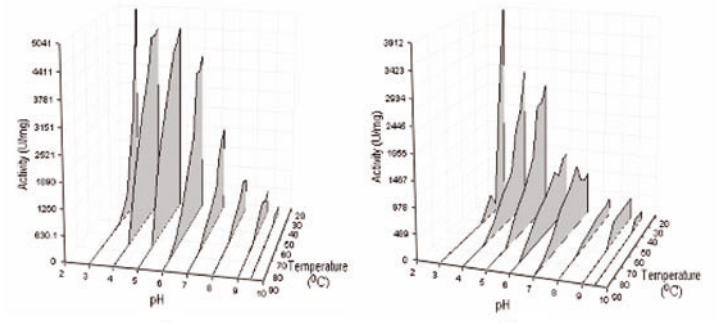


Figure 4. Activities of purified HRP (a) and HRP-dextran aldehyde conjugate (b) with molar ratio n_{HRP}/n_{DA} 1/10, depending on pH, at different temperatures. Activities were determined with 60 minutes incubation at working temperatures.

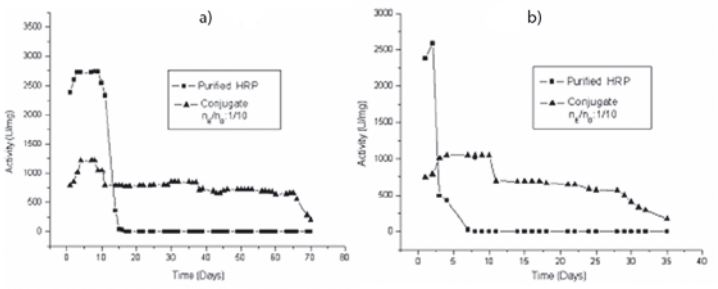


Figure 5. Storage lifetimes of purified enzyme and the conjugate with n_{HRP}/n_{DA} 1/10 at (a) +4°C and (b) room temperature (Three consecutive activity measurements were carried out for each day and were averaged).

with dextran 188 kDa showed stability against temperature, but had lower activities compared to the conjugates with 75 kDa dextran. Again, the conjugate n_{HRP}/n_{DA} 1/10 with 188 kDa dextran was also the best or better one in all conjugates prepared with 188 kDa dextran. Conjugation with 17.5 kDa dextran also supplied thermal stability but less than conjugation with 75 kDa dextran.

When the activities of pure enzyme and the conjugate(n_{HRP}/n_{DA} 1/10, 75 kD dextran) were determined at different pHs with 60 minutes incubation (Figs. 4a and b), only the conjugate has activity over 70°C at pH 7.

In addition, storage lifetimes of purified enzyme and the conjugate with n_{HRP}/n_{DA} 1/10 were studied for +4°C and room temperature (Fig. 5a, b) at pH 7. Purified enzyme lost its activity after 14 days at +4°C and after 4 days at room temperature. Whereas, conjugate was stable, showing good activity values for 65 days at +4°C and 27 days at room temperature. As a result, this conjugate had a longer storage lifetime compared to purified and purchased enzymes at +4°C and room temperature; which is considered as a good feature for usage in practice.

4. Conclusion

We obtained the stable horseradish peroxidase-dextran aldehyde conjugates against temperature and pH for the first time. Multipoint covalent bonding of dextran to horseradish peroxidase caused the formation of a conjugate whose thermal stability was increased especially at pH 7. Optimizing the procedure by using different molar weighted dextrans in different molar ratios provided to obtain the conjugate (n_{HRP}/n_{DA} 1/10) with higher stability. In addition, long storage lifetimes at +4°C and room temperature for this conjugate were obtained.

Acknowledgments

Authors wish to commemorate this study to beloved professor Mamed Mustafaev.
This research was supported by grants from T.R. Prime Ministry State Planning Organization (Project No. 25-DPT-07-04-01) and Yildiz Technical University BAPK (The Production of Medical Biosensors: Project No. 27-07-04-01).

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