Carrier Free Cross-linked Peroxidase Aggregates: Synthesis and Characterization

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Abstract

In this study, cross-linked Horseradish Peroxidase (HRP) aggregates were prepared. Bovin Serum Albumin (BSA), a proteic feeder, and glutaraldehyde, a bifunctional reactive, a crosslinker agent has also been used. The initial enzyme concentration (0.05 mg/ml), BSA (5 mg) and glutaraldehyde (GA) (4 µl/ml) amounts were optimized and maximum immobilized activities were obtained. The kinetic parameters of free and immobilised enzyme have been calculated by using Lineweaver-Burk plots. The saturation constant (K_m) and the maximum reaction rate (V_m) of free enzyme were found as 2.33 mM and 0.670mmol/l.sec, respectively. The apparent kinetic parameters (Km_{app} and Vm_{app}) of cross linked enzyme aggregates have been calculated as 33.4 mM and 0.127 mmol/l.sec, respectively.

Key Words: Cross-linked enzyme aggregates, horseradish peroxidase, bovine serum albumin, proteic feeder.

INTRODUCTION

In the second half of the last century, numerous efforts were devoted to the development of various carrier bound immobilised enzymes with the aim of facilitating their use in continuous processes [1,2]. Improved enzyme performance, such as activity, stability and selectivity, can often be achieved by enzyme immobilisation [3-5].

In general, the techniques currently used for creation of robust immobilised enzymes, which meet both catalytic requirements and non-catalytic requirements expected for a given process. These are all characterized in that a combined method is used to solve problems that are unsolvable by the straightforward method.

The development of robust immobilised biocatalysts that are stable over a broad range of pH and temperature and are tolerant to organic solvents is a major challenge in industrial biocatalysis [6]. A major breakthrough in this field was the development of cross-linked enzyme crystals (CLECs), biocatalysts which combine the features of essentially pure protein with high specific activity and high tolerance to organic solvents [7]. However, the preparation of CLECs requires the crystallization of the enzyme prior to cross linking.

Also worthy of note is that in the last few years of the 1990's it was discovered that not only enzyme crystals but also physical enzyme aggregates could

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be cross-linked to form catalytically active insoluble immobilised enzymes, nowadays known as CLEAs. It has recently been found that industrially robust CLEAs, with greater activity both in organic solvents and in aqueous media, can be prepared. Free CLEAs are usually prepared by chemically cross-linking the physically enzyme aggregates by using appropriate aggregation agents such as those widely used in non-denaturing protein separation [8]. CLEAs can be prepared with greater mechanical stability and tailor-made size. In principle, CLEAs are applicable to any reaction system, reactor configuration and reaction medium. Recently studies enclose the development of CLEAs of abroad range of enzymes, size control, new aggregation methods and new cross-linkers to construct a flexible technology platform for screening and designing robust CLEAs for broad applications [9].

The formation of such aggregates is generally attributed to intramolecular and intermolecular cross-links introduced in the protein molecule. Considerable information is already available about such aggregates which have been formed from various kinds of enzymes (penicillin acylase, lipase, etc). A member of the oxidoreductases group, peroxidases (EC 1.11.1) catalyzes oxidation-reduction reactions. Peroxidases have so many application areas such as biosensor development, clinical diagnosis, and bioremediation [10,11].

In this study, cross-linked Horseradish Peroxidase aggregates (CLEA) were prepared. The immobilization process was carried out by using a proteic feeder and crosslinker agent. The initial enzyme concentration, BSA and glutaraldehide amounts were optimized and maximum immobilized activities were obtained. The kinetic parameters of free and immobilised enzyme have been calculated.

MATERIALS and METHODS

Materials

HRP Type IV (Sigma Chem. Co., USA), Bovine Serum Albumin (Sigma Chem. Co., USA), $Na_2HPO_4.2H_2O$ (Riedel-de Haen, Sigma-Aldrich, Germany), $NaH_2PO_4.2H_2O$ (Riedel-de Haen, Sigma-Aldrich, Germany) were purchased.

Preparation of CLEAs

The object of the study consists of producing cross-linked enzyme aggregates. Chemical aggregates of horseradish peroxidase were prepared as described below. Bovine serum albumin (BSA) was used as proteic feeder to facilitate (accelerate) enzyme aggregate formation. The enzyme (80 μl) was dissolved in distilled water. BSA was added and again dissolved by constant stirring (100 rpm) for 5 min. After glutaraldehyde addition, the medium pH was adjusted to pH 7 with a suitable amount of 0.1 M NaOH and final volume was made up to 1 ml with distilled water. After incubation at 25°C for 2 h (with constant stirring for the first 15 min), the reaction mixture was left overnight at 4°C. The aggregated protein was collected by centrifugation at 7,000 *g* for 15 min at 4°C. The supernatant was gently discarded. Distilled water was added and homogenized gently with a glass rod. The aggregates washed several times in order to remove unreacted glutaraldehyde and BSA. The washing was repeated extensively with distilled water until the supernatant was free of protein and enzyme activity. The existence of protein in the supernatant was examined by modified Lowry total protein determination method.

Some synthesis parameters of the CLEAs were examined to determine the optimum conditions. The selected parameters and their working ranges were the initial enzyme concentrations as 0.01, 0.025, 0.050, 0.075, 0.100 mg/ml, the BSA amount as 1, 5, and 7.5 mg, and glutaraldehyde concentrations as 2, 4, 6 μl/ml [12].

Measurement of native and CLEA activity

The activities of native and CLEA of HRP were measured according to a previous method [13] with some modification. 0.1 ml of native HRP or 0.1 ml of CLEA was added to 2.4 ml of an assay mixture containing 0.1 mol l^{-1} phosphates or acetate buffer and 12.8 mmol l^{-1} of pyrogallol. The reactions were started with the addition of 0.5 ml of 5.0 mmol l^{-1} H_2O_2 solutions. For native HRP, the reaction product, puppurogallin was measured at 420 nm, 20 seconds after H_2O_2 addition. The reaction of the CLEA of HRP was tested at time intervals from 1 to 3 min. The Michealis-Menten constants K_m and V_m values were determined by using Lineweaver-Burk plot.

An enzyme unit is the enzyme amount which catalysis pyrogallol to 1 mg purpurogallil at 25°C and pH 6.0 within 20 sec. One purpurogallin unit is also defined as the amount of enzyme which converts 12 μmol of H_2O_2 to purpurogalline at 25°C and pH 6.0 where activity unit is 181 purpurogallil unit/mg enzyme amounts.

RESULTS AND DISCUSSIONS

Preparation of CLEAs

Soluble enzyme molecules can be made to form physical aggregates. It is possible to form aggregates by changing the hydration state of enzyme molecule or by altering electrostatic constant of the solution by adding appropriate aggregation agents [14]. The insoluble chemical aggregates of horseradish peroxidase enzyme were prepared by cross-linking of the enzyme with glutaraldehyde in the presence of BSA as proteic feeder. In this part of the research visual results of formation rates of CLEAs were investigated during aggregation procedure. By adding aggregation agents at these conditions soluble enzyme aggregates associate together to the extent that they precipitate as insoluble aggregates with native enzyme conformation. Table 1 publishes the results of the visual observations and also shows definitions of the scores used for the experiments done with the aggregation level.

Table 1. The observation of aggregate formations*.

Variables	Scores
Initial Enzyme Concentration (mg/ml)	
0.010	+
0.025	+
0.050	++
0.075	++
0.100	++
BSA Amount (mg)	
1	+
5	++
7.5	+++
GA Concentration (μl/ml)	
2	++
4	++
6	+

*High aggregation (+++); Medium aggregation (++);
Low aggregation (+)

As it can be seemed from the table that very little insoluble aggregate formation were observed at low enzyme concentrations. The aggregate formation increased at 0.050 mg/ml and higher enzyme concentrations. The aggregate formation was also increased by increasing of BSA amount. At low glutaraldehyde concentrations, good aggregate formation was obtained but low aggregation was obtained at high GA concentration. The visual observations indicate that initial enzyme and GA concentrations and BSA amount are important parameters in aggregate formation.

Carrier-free enzyme immobilisation parameters

The optimum initial enzyme concentration

Initial enzyme concentration was an important criteria to affect activity of immobilised enzyme. The enzyme activity of immobilized enzyme is another crusial effect to be tested. So, enzyme solutions have prepared with different concentrations (0.010, 0.025, 0.050, 0.075, 0.100 mg/ml) to investigate the effect of initial enzyme concentration on CLEA forming. BSA and GA concentrations were kept constant as 5 mg and 0.4 % v/v. The activities of CLEAs per immobilized enzyme amounts were determined at each initial enzyme concentration. The results of this group of experiments are given in Figure 1.
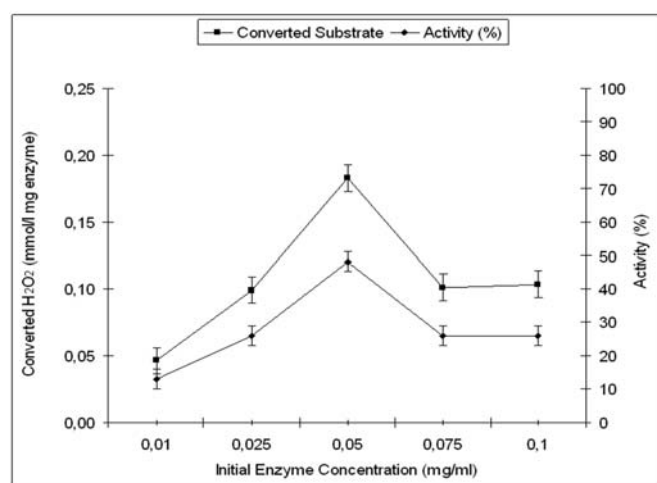

Figure 1. Effect of initial enzyme concentration on enzyme activity.

As it is seen from Figure 1, enzymatic activity of CLEAs increased with increasing enzyme concentration until 0.05 mg protein/ml. After mentioned initial enzyme concentration, enzymatic activity decreased probably because of accumulation of enzyme molecules. Active sites of enzyme molecules might be restricted by random bonding of molecules and this may lead to decrease of activity.

The optimum BSA amount

Another parameter which affects the immobilisation efficiency was the optimum BSA concentration. BSA was used as proteic feeder to prepare CLEAs of horseradish peroxidase enzyme [11,12]. The proximity of dissolved enzyme molecules was changed by adding BSA to form physical aggregates which after cross-linking are termed CLEAs. The effect of BSA concentration on CLEAs forming was also investigated in some previous studies [11,12]. Cross-linked enzyme aggregates were prepared in the presence of different albumin concentrations to investigate the proteic feeder concentration effect. Three different BSA concentration value (1, 5, and 7.5 mg) were used for this purpose. Initial enzyme and glutaraldehyde concentrations were kept constant as 0.05 mg/ml and 0.4 % v/v, respectively. The results of this section were shown in Figure 2. Within this range of employed proteic feeder concentration, there is an optimum concentration of albumin for obtaining most active CLEA with BSA.

In fact there is an optimum range of BSA amount. There are not enough free amino groups (contributed by BSA) to prevent excessive cross-linking at lower amount of this range. At higher than optimum range of BSA amount, the free amino groups of BSA compete with free amino groups of peroxidase and prevent the necessary cross-linking of peroxidase molecules. As it is seen from Figure 2; 5 mg BSA amount was optimum amount for HRP and for the below and beyond BSA amounts, there were not sufficient activity values, as also pointed out in an earlier study [11].

The optimum glutaraldehyde concentration

Optimization of initial enzyme and BSA amount is not sufficient to reach the maximum CLEA activity. Cross-linking agent, glutaraldehyde concentration optimization is also very important criteria for CLEA activity optimization. For this reason, effect of glutaraldehyde concentration variations on activity was investigated and the results were shown in Figure 3. The bifunctional glutaraldehyde molecules were used for crosslinking of the physically aggregated enzyme molecules. Glutaraldehyde
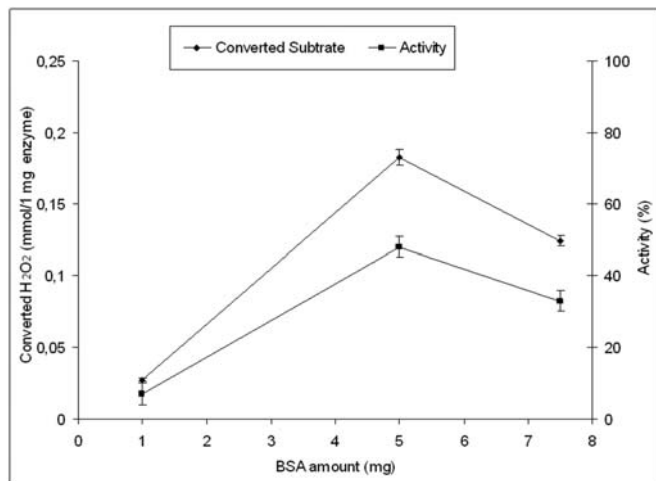
Figure 2. Effect of BSA amount on enzyme activity.

concentration varied as 2, 4, 6 µl GA/ml (2,4,6 % v/v) .

Initial enzyme and BSA concentrations were kept constant as 0.05 mg/ml and 5 mg, respectively. According to the Figure 3, variation of glutaraldehyde concentration is fairly effective for CLEA activity. When glutaraldehyde concentration was 4µl/ml CLEA activity has the maximum value. The activity values for low and high GA concentrations were lower than the activity obtained for 4µl/ml GA. The low enzyme activity when 2 % v/v was used may greatly due to the loss of enzyme molecules uncross-linked. On the other hand, high concentration cross-linking agent again leads to low apparent enzyme activity which may be the results of highly crosslinking network structure as mentioned at the earlier studies [11,15].
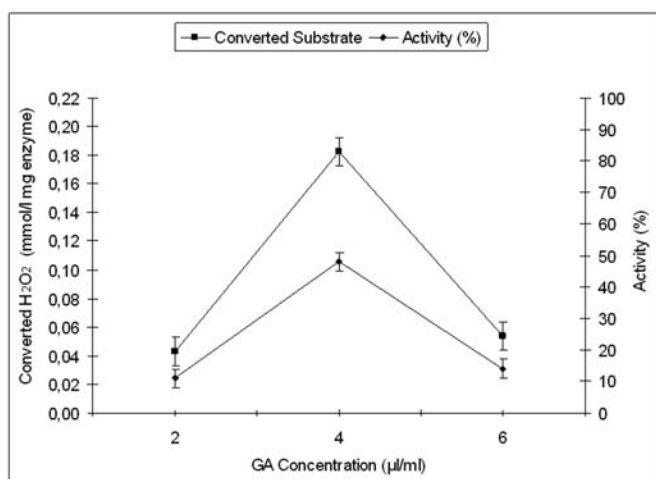

Figure 3. Effect of glutaraldehyde concentration on enzyme activity.

Determination of Michealis-Menten Constants

The effect of immobilisation on the kinetic parameters, K_{mapp} and V_{mapp} were also studied. Line weaver-Burk plots were drawn to determinate the kinetic parameters. The calculated kinetic values for free and immobilized enzyme are shown in Table 2. Figure 4a and 4b presents the Line weaver-Burk plots for both native and CLEA of HRP.

Table 2. Kinetic parameters of free and CLEAs of HRP determined by using Line weaver-Burk method.

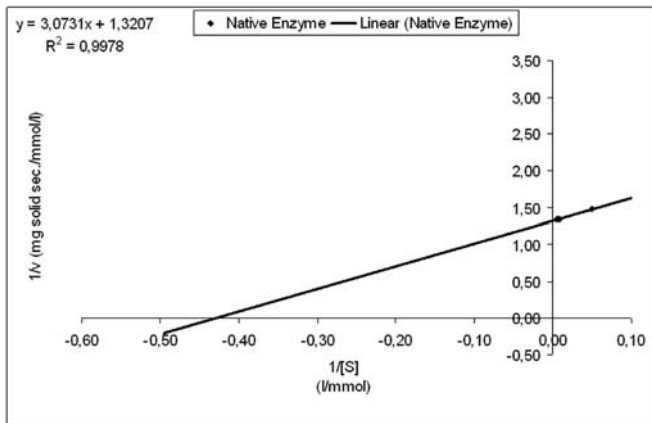|	Km (mmol/l)	Vm (mmol/l.s)
Free enzyme	2.33	0.760
Carrier-free Immobilised	33.4*	0.127*

* apperent

There was an appreciable difference for K_m values between free enzyme and CLEA. The possible reason of this difference could be based on the molecular three dimensional changes which had realized in course of aggregation procedure. So, the substrate affinities of CLEA decrease as a result of increasing of Km_{app} constant. But when we compare Km values of CLEA of HRP with our group's earlier study [16] carrier bound HRP, K_m value of carrier bound enzyme of HRP was found as 96.16mmol/l, whereas K_m value of CLEA was found 33.4mmol/l. It was seen that the substrate affinity of CLEA is three times greater than substrate affinity of carrier-bound immobilised enzyme. Similar opinions were mentioned at a study in literature [10]. The maximum apparent enzyme reaction rate was also decreased as a result of low substrate affinity compared with that of free enzyme.

The optical micrographs of CLEAs prepared under optimum conditions were shown in Figure 5a and b. The micrographs indicate a compact, amorph, non ductile and non spherical network structure.
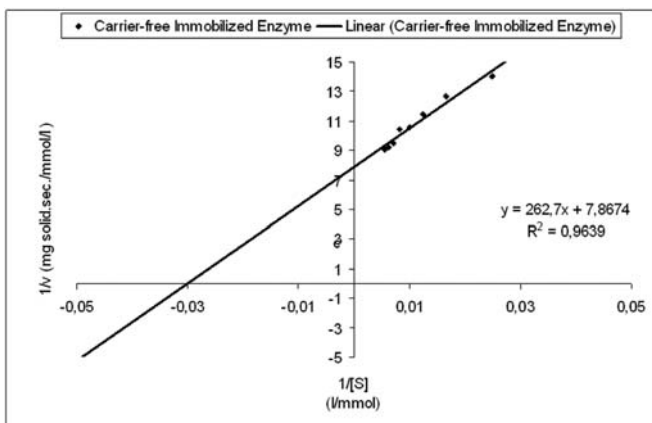
CONCLUSION

In this work, a novel cross linked aggregate of HRP enzyme has been shown to have greatly enhanced enzymatic activity. Raison of this high enzymatic acivity is the carrier-free immobilised enzyme systems with non catalytic mass compared with carrier-bound immobilised enzyme systems. The carrier-free immobilised enzyme systems also offers different advantages compared with carrier-bound immobilised enzyme systems. A potential area for future work involves improving the activity retention of the enzyme immobilization.

(a)

(b)

Figure 4. Lineweaver-Burk plots. (a) Native enzyme, (b) Carrier free immobilized Enzyme.

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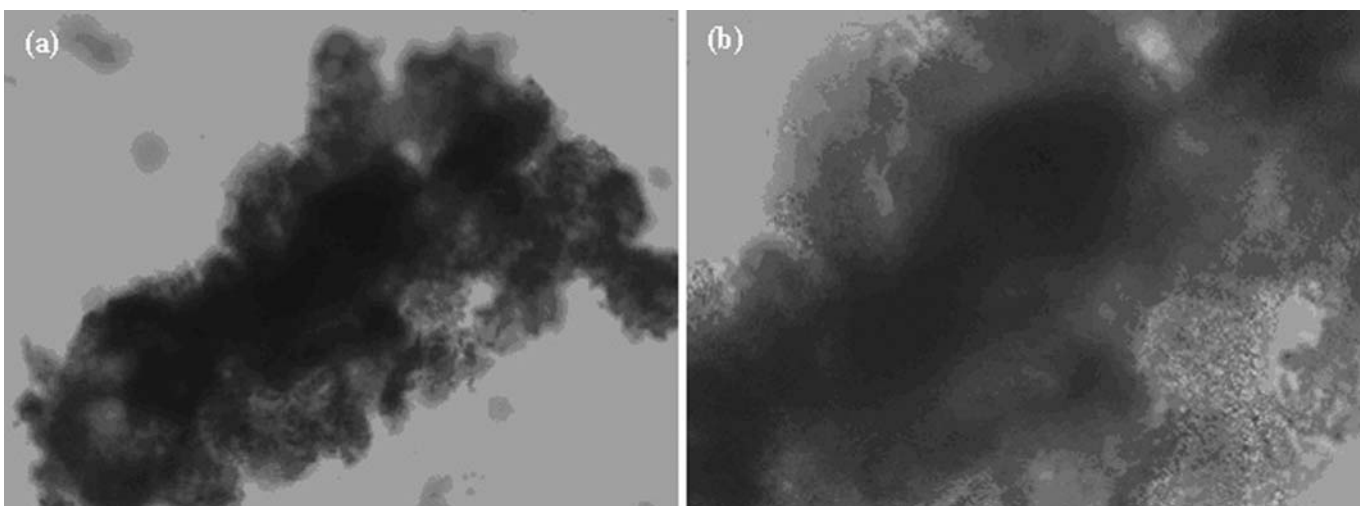
Figure 5. Optical micrograph of cross-linked POD aggregates. (a) x40 and (b) x60 magnification.

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