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Stabilization of horseradish peroxidase in aqueous-organic media by immobilization onto cellulose using a cellulose-binding-domain

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Abstract

A fused protein consisting of a cellulose-binding domain (CBD) and horseradish peroxidase (HRP) was bound to cellulose beads and evaluated in aqueous-organic solvent systems. The CBD–HRP fusion protein containing two different functionalities, a catalytic domain and a binding domain, preserved both capabilities in this non-conventional environment. A six-fold increase in the half-life of the enzyme in buffer resulted from immobilization onto cellulose via CBD. The immobilized enzyme was also more stable than the native enzyme in increasing concentrations of acetone (0–92%). There was a general decrease in activity as the solvent concentration in the mixture increased (in all solvent types: THF, acetone, acetonitrile and ethanol). However, the immobilized enzyme was at all times more active than the soluble enzyme forms. The thermostability of the enzyme in buffer, at 40–60 °C, was also improved by immobilization. The soluble CBD–HRP fusion protein exhibited greater stability (both to organic solvents and temperature), but lower activity, in comparison with the native HRP. This work demonstrates for the first time the use of a cellulose-bound CBD-enzyme as a catalyst in aqueous-organic solvent media.
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1. Introduction

In recent years there has been a considerable increase in the use of enzymes as industrial catalysts [1,2]. However, the practical use of enzymes often requires working under denaturing conditions, such as elevated temperature, to increase productivity, and an aqueous-organic environment, to shift the reaction equilibrium toward desired products, and to enhance substrate solubility. Enzyme stabilization is, therefore, of major importance in applied biocatalysis [3,4]. Immobilization is one of the most common approaches to enzyme stabilization, and >10,000 papers and patents have been published on this subject since the early 1960s [4–6]. Bioaffinity-based enzyme immobilization is a method that utilizes the high affinities between biomolecules and their ligands. Such an immobilization procedure usually results in enzyme preparations that exhibit high catalytic activity and improved stability against denaturation [7]. Recently, developments in the areas of recombinant DNA technology and protein-engineering, enable fusion of enzymes lacking innate binding affinity, to appropriate polypeptide domains that can bind to various types

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of supports. Cellulose binding domain (CBD) is an example of such a polypeptide widely used as an affinity tag for the purification of proteins [7–9].

Cellulose binding domains (CBDs) are essential components of a wide variety of cellulose and hemicellulose degrading enzymes [10,11]. A common feature of all CBDs is that all have affinity for cellulose, but they do not have any hydrolytic activity. They appear to play a multiple role in hydrolysis of crystalline cellulose. When the cellulases or cellulosomes approach the plant cell surface, their CBDs mediate binding of the enzyme-complex to cellulose [10,11]. In addition to their more obvious role as a targeting vehicle, it has been proposed that CBDs may mediate the non-hydrolytic disruption of cellulose fibers, thereby facilitating subsequent enzymatic degradation by the catalytic domains [12]. The strong affinity of CBDs for cellulose makes them attractive candidates for various technological applications [13]. The most commonly studied and first commercial application is the use of CBDs in fusion proteins as tags for affinity purification or immobilization [14,15]. Another commercial application is in the laundry detergent industry. CBD is fused to the hydrolytic enzymes found in detergents, to help to target them to the cellulose in the textile [16,17]. In such applications, the use of CBDs offers many industrially attractive advantages. Since the CBDs adsorb spontaneously to cellulose from almost any solution, very little pre-treatment of the samples is required prior to immobilization. Moreover, cellulose is an inexpensive, chemically inert material, which is safe for use even in food or pharmaceutical applications. Many cellulose matrixes with different properties are commercially available. The main feature of using CBDs is the ability to purify and immobilize the fused protein in a single step [8,9].

Several examples of using CBD-fusion enzymes for catalytic purposes can be found in the literature. Atrazine chlorohydrolase was fused to CBD, immobilized onto cellulose and used for removal of atrazine, a common herbicide found in water reservoirs [18]. The immobilized CBD–atrazine–chlorohydrolaze was found to be a useful catalyst for dechlorinating the pollutant, while retaining its activity for 5 days. In another example, heparinase was fused to CBD for the continuous depolymerization of heparin to low molecular weight heparin oligosaccharides [19]. β-Glucosidase was also immobilized to cellulose of various kinds via CBD and showed long term hydrolytic activity in a column reactor [20]. A recent example of catalysis using a CBD-enzyme complex is the hydrolysis of organophosphorus compounds, which are amongst the most toxic substances known [21]. Purification and immobilization of CBD-organophosphorus hydrolase (OPH) onto a variety of cellulose matrixes, was easily achieved in a single step and paraoxon was efficiently degraded by the immobilized CBD–OPH. The kinetic properties of CBD–OPH, immobilized onto cellulose or free in solution, were shown to be similar to the wild-type OPH enzyme. Levy et al. [22] have recently reported that CBD fused to horseradish peroxidase (HRP) may be used for oxidation of *p*-bromophenol, a toxic pollutant in industrial wastewater. Thus, it seems likely that CBD-fusion proteins have considerable potential as robust catalysts for organic synthesis. However, this route has been only slightly exploited to date.

It should also be emphasized that all the applications described above have been restricted to aqueous systems only, and there are no descriptions of studies carried out in non-conventional media. In this paper, we describe the first attempt of using a CBD fusion protein as a catalyst in such a non-conventional medium. The influence of various types of water-solvent mixtures, on the activity and stability of CBD–HRP in soluble and immobilized form, is described.

2. Experimental

2.1. Materials

p-Anisidine and hydrogen peroxide (30% solution) were purchased from Aldrich (Rehovot, Israel). Cellulose beads (Sigmacell 50) were from Sigma (Rehovot, Israel). Protein was determined using the BCA (bicinchoninic acid) protein assay reagent kit (Pierce, IL, USA). Activity measurements of HRP were performed using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate (TMB kit, Pierce, IL, USA).

All other solvents and reagents were obtained commercially and were of analytical grade unless otherwise stated.

Spectroscopic measurements were performed using a Pharmacia LKB Ultrospec III spectrophotometer (Pharmacia).

2.2. Construction, expression and refolding of pETHRP and pETCBD-HRP

Using standard DNA manipulations techniques [23], a synthetic HRP gene in *Escherchia coli* codon usage (cloned *Nde*I/*Bam*HI in pUC19: pUC19-HRP), EC 1.11.1.7 (R&D Systems Inc., Minneapolis, MN) was cloned (*Nde*I/*Bam*HI) into predigested (*Nde*I/*Bam*HI) pET29a(+) resulting in pET29HRP. In order to fuse the cellulose-binding domain from *Clostridum cellulovoranse*(CBD_{Clos}) to HRP, the CBD_{Clos} gene from pETCBD-180 [19] was digested (*Avr*II/*Bam*HI) and ligated with an *hrp* gene from pUC-HRP that was predigested (*Nde*I/*Bam*HI). The fused gene was then inserted into pET29a(+) vector (Novagen Inc., Madison WI) to give pETCBD-HRP. The integrity of the clone was confirmed by sequencing. HRP and CBD–HRP were over expressed in *E. coli* BL21 (DE3) harboring the pETHRP or pETCBD-HRP plasmids. Overexpression was performed in TB medium (12 g Bacto trypton, 24 g Bacto yeast extract, 4 ml glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) containing 50 μg/ml kanamycin for pETHRP or 50 μg/ml ampicillin for pETCBD-HRP at 37 °C to an OD_{600} of 0.6, after which 1 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG) was added. Following overnight incubation under the same condition, the cells were harvested by centrifugation and washed twice in 20 mM Tris pH 8.0. The bacterial pellet was resuspended in 30 ml lysis buffer (20 mM Tris pH 8.0, 1 mM EDTA, 0.1% v/v Triton X100, 10 μg/ml lysozyme, 5 μg/ml DNaseI, 0.5 mM phenyl methyl sulphonyl fluoride) and incubated at 37 °C for 30 min. Inclusion bodies were collected by centrifugation at 15,000 × *g* for 10 min followed by four washes in 20 ml 20 mM Tris pH 8.0 containing 1 mM EDTA and 1% Triton X100. Refolding of inclusion bodies was preformed according to Levy and Shoseyov [24] with the following changes. Inclusion bodies were dissolved in denaturating solution (4.5 M urea, 40 mM Tris base pH 11.3 and 1 mM cysteine and 0.2 mM hemin) to give a protein concentration of 0.1–0.3 mg/ml protein, as determined via a micro BCA protein assay reagent kit (Pierce, Rockford, IL). The denatured proteins were dialyzed overnight at 4 °C against 20 mM Tris base pH 8.5 containing 2 M urea, 0.2 mM hemin, 5 mM $CaCl_2$ and 150 μM oxidized glutathione. The refolded protein solution was centrifuged 16,000 × *g* for 15 min to precipitate misfolded protein and then analyzed by 12.5% SDS-PAGE.

2.3. Stability of cellulose bound CBD_{Clos} in organic solvents

CBD_{Clos} (kindly provided by CBD-Technologies, Rehovot, Israel) at a protein concentration of 2.5 mg/ml was incubated with cellulose beads (Sigmacell 50) at room temperature. After 1 h the cellulose was filtered and washed three times with phosphate buffered saline (PBS). A total of 5 mg of beads were placed in polypropylene microtubes with 1.5 ml of different solvents at 50 °C or buffer at 4 and 50 °C. Following 15 h of incubation the solvents were removed and the cellulose was washed three times with PBS buffer. Bound CBD was determined using the Lowry method [25]. Lowry assay was also performed on cellulose samples that were not bound with CBD and were treated under the same conditions of solvent and temperature.

2.4. Binding of CBD–HRP onto cellulose

A total of 1 ml of CBD–HRP solution in 20 mM Tris base buffer, pH 8 (100 μg protein) was mixed with 1.5 g cellulose beads (Sigmacell 50) at constant rotation head-over-head at room temperature for 1 h. The mixtures were centrifuged at 15,000 × *g* for 5 min. The precipitate was washed with 10 ml of 20 mM Tris base buffer pH 7.5 containing 1 M NaCl and centrifuged again. Two more washings were performed using the same buffer without NaCl. The obtained wet cellulose was kept at 4 °C or dried as follows: (a) lyophilized, (b) lyophilized with 1 g sucrose (10% w/v solution), (c) dried in a dessicator at room temperature for 24 h. Bound protein was determined by adding 2 ml of BCA reagent to 25 mg of cellulose–CBD–HRP followed by incubation at 37 °C for 30 min. The enzyme was filtered and the absorbance measured at 562 nm. For measurements of soluble protein, 0.1 ml of HRP or CBD–HRP were used in the assay.

2.5. Stability measurements of HRP in acetone–buffer mixtures

Acetone–buffer (Tris buffer 20 mM pH 7.5 containing 4 mM $CaCl_2$) mixtures at the following

concentrations were prepared: 0, 20, 40, 60, 80, 92% acetone. A total of 1 ml of solvent mixture containing 0.12 μg/ml HRP or 8 μg/ml CBD–HRP was incubated in 40 °C. At different time intervals, 20 μl aliquots were removed and assayed for residual activity. For stability measurements of the immobilized enzyme, 20 vials containing 1 ml solutions with 4 mg enzyme (lyophilized with sucrose) were incubated at 40 °C. At different time intervals, a vial was removed from the incubator, the solvent solution discarded and the enzyme analyzed for residual activity. The measurements were performed in duplicates. The residual activity of HRP was determined using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate [26]. A total of 0.5 ml of TMB was mixed with 20 μl enzyme solution or 4–8 mg of cellulose bound enzyme, for 3 min at room temperature (in glass vials containing a magnetic stirrer). The reaction was stopped with 0.5 ml sulfuric acid (2 M H_2SO_4) and the absorbance was measured at 450 nm in glass cuvettes (the solution containing the immobilized enzyme was filtered prior to measurement).

2.6. Thermostability measurements of HRP

Thermostability measurements were performed in a similar way, in buffer (Tris buffer 20 mM pH 7.5 containing 4 mM $CaCl_2$) with incubation in 40, 50, 60, and 70 °C.

2.7. Activity measurements of HRP

The activity assay of HRP was based on the oxidation of *p*-anisidine with hydrogen peroxide (modification of the method used by Kazandjian et al. [27]) The reaction mixture (10 ml volume) contained 1 mM *p*-anisidine and 0.2 mM H_2O_2 in different solvent–buffer (Tris buffer 20 mM pH 7.5) ratios: 0, 20, 40, 60, 80, and 100% solvent. The solvents used were THF, acetonitrile, acetone and ethanol. The reactions were started by adding the enzyme, and the increase in absorbance was measured at 460 nm. The concentration of enzyme in the reactions mixture was such that the absorbance did not exceed 0.8 OD. For HRP 0.08 μg/ml, CBD–HRP 3.8 μg/ml, cellulose bound CBD–HRP (lyophilized with sucrose) 30 μg/ml. In the case of immobilized enzyme, aliquots were removed from the reaction, at 2–5 min intervals, filtered and monitored. The assay was performed in duplicates.

3. Results and discussion

3.1. Stability of cellulose–CBD in organic solvents

It is now believed that enzymes are catalytically active in organic solvents because they remain trapped in the native conformation [28]. HRP has been shown to work in organic solvents [27,29], as well as in aqueous-organic solvent mixtures [30–32]. To the best of our knowledge, the use of CBD in organic solvents has not yet been reported. Before testing the activity of the bound enzyme, it was of importance to check the stability of the cellulose–CBD conjugate itself in various organic solvents. For this purpose, CBD_{Clos} (from *Clostridum cellulovorans*) was bound to cellulose beads (Sigmacell 50) and incubated for 15 h in different solvents at 50 °C. The amount of bound protein was determined before and after incubation and the residual binding is presented in Table 1. The general

Table 1
Stability of cellulose bound CBD in organic solvents

Solvent	Bound CBD (μg/mg cellulose)	Relative residual binding[a]
Buffer, 4 °C	18.94	1.00
Buffer, 50 °C	18.18	0.96
Dichloromethane	16.27	0.86
1,4-Dioxane	17.60	0.93
Butanol	18.42	0.97
THF	16.21	0.86
DIPE	18.20	0.96
1-Methyl-2-pyrrolidon	17.20	0.91
Ethyl acetate	16.04	0.85
Cyclohexane	17.05	0.90
Toluene	16.38	0.86
Carbon tetrachloride	18.06	0.95
Acetonitrile	19.01	1.00
Methyl isobutyl ketone	18.50	0.98
Hexane	18.77	0.99
Chloroform	17.85	0.94

CBD at a protein concentration of 2.5 mg/ml was incubated with Sigmacell 50 cellulose beads at RT for 1 h. The cellulose was washed three times with PBS buffer. A total of 5 mg cellulose were placed in microtubes with 1.5 ml of solvent at 50 °C. After 15 h, the cellulose was washed with PBS and bound CBD was determined.

[a] Bound CBD in solvent/bound CBD in buffer 4 °C.

conclusion is that CBD_{Clos} remained bound to cellulose in essentially all solvents tested. CBD bound to a cellulose membrane gave similar results (not shown). It was established that CBD remained bound to cellulose in common organic solvents, and the activity of conjugated HRP could, therefore, be evaluated.

3.2. Binding of CBD–HRP onto cellulose beads

CBD–HRP was immobilized onto cellulose beads and dried using various techniques. Activity measurements in the remaining supernatant revealed that 95% of the active enzyme was bound to the cellulose (via CBD). A relatively low loading of 0.1 mg protein/g cellulose was used, so as to achieve convenient conditions in the spectrophotometric assay of activity. It is known that higher levels of loading of up to 20 mg/g can be achieved with this type of cellulose [22]. Drying of the immobilized enzyme was necessary prior to use in organic solvents, and the results in Table 2 demonstrate that the method of drying has a crucial effect on the specific activity.

When drying is essential, it is evident that the best means of preserving the activity is by lyophilization with sucrose. This is in accordance with previous work demonstrating that lyoprotectants such as sorbitol enhance enzymatic activity by diminishing enzyme denaturation upon lyophilization [33,34]. In addition, the lyophilized enzyme (C) was found to lose 60–70% of its activity within 3 weeks of storage at 4 °C, whereas the enzyme lyophilized with sucrose (D), lost only 5% of its activity under these conditions. For further experiments, we used cellulose bound CBD–HRP lyophilized from a 10% sucrose solution.

Table 2
Effect of drying method on the activity of cellulose-bound CBD–HRP

Treatment		Specific activity (μM/min mg protein)
A	Not dried (wet beads)	2790
B	Dried for 24 h in a dessicator at RT	80
C	Lyophilized	410
D	Lyophilized with sucrose (10% w/v solution)	1840

1 ml CBD–HPR (100 mg protein) in buffer was mixed with 1 g sigmacell beads at room temperature for 1 h. Centrifugation at 15,000 rpm for 5 min. Washing of the pellet with 1 M NaCl–Tris buffer 20 mM pH 7.5 and a second washing with Tris buffer alone. Activity measurements were performed using the TMB kit. Protein measurements were done using the BCA kit.

3.3. Stability of HRP in acetone–buffer mixtures

The native structure of a protein is usually regarded as the conformation exhibited by proteins within the cellular environment or at their maximum biological activity [35]. Protein denaturation is a process involving a major or minor change of this three dimensional structure, without altering the amino-acid sequence. Hydrophilic solvents are known to facilitate unfolding by displacing water from the hydration shell of the proteins [36], and through dielectric effects on protein dynamics [37]. Unfolding is considered to be the rate-limiting step in irreversible deactivation of proteins, and therefore, stabilization of protein molecules refers preventing this change and preserving the native structure [3,38]. Immobilization is the most widely used method for enzyme stabilization [4]. Ideally, the immobilized enzyme will exhibit improved catalytic performance. Stabilization by this method is attributed to the more rigid conformation of the immobilized enzyme as compared with the free form [39].

Three forms of HRP (soluble HRP, soluble CBD–HRP and cellulose bound CBD–HRP) were incubated in acetone–buffer mixtures and analyzed for residual activity (E/E_0). The denaturation profiles presented in Fig. 1 agree with the simple exponential model $E/E_0 = \exp(-K_d t)$ described for HRP by others [31,40]. The correlation coefficient of the model to the experimental data was generally >0.960. Table 3 represents a summary of the inactivation constants (K_d) and half-lives ($t_{1/2}$, the time required for the enzyme to lose half of its original activity) that were derived from the mathematical model for the three enzyme-forms. Azevedo et al. [31] reported a similar profile for the soluble HRP but described a two-stage deactivation model for a covalently immobilized HRP. According to this model an activated intermediate species is formed prior to deactivation. Our results show that in buffer, as well as in all acetone–buffer mixtures, the immobilized enzyme was more stable than the free enzymes ($t_{1/2}$ was always higher).

All of the HRP forms lost their stability with increasing concentrations of acetone as reflected by the

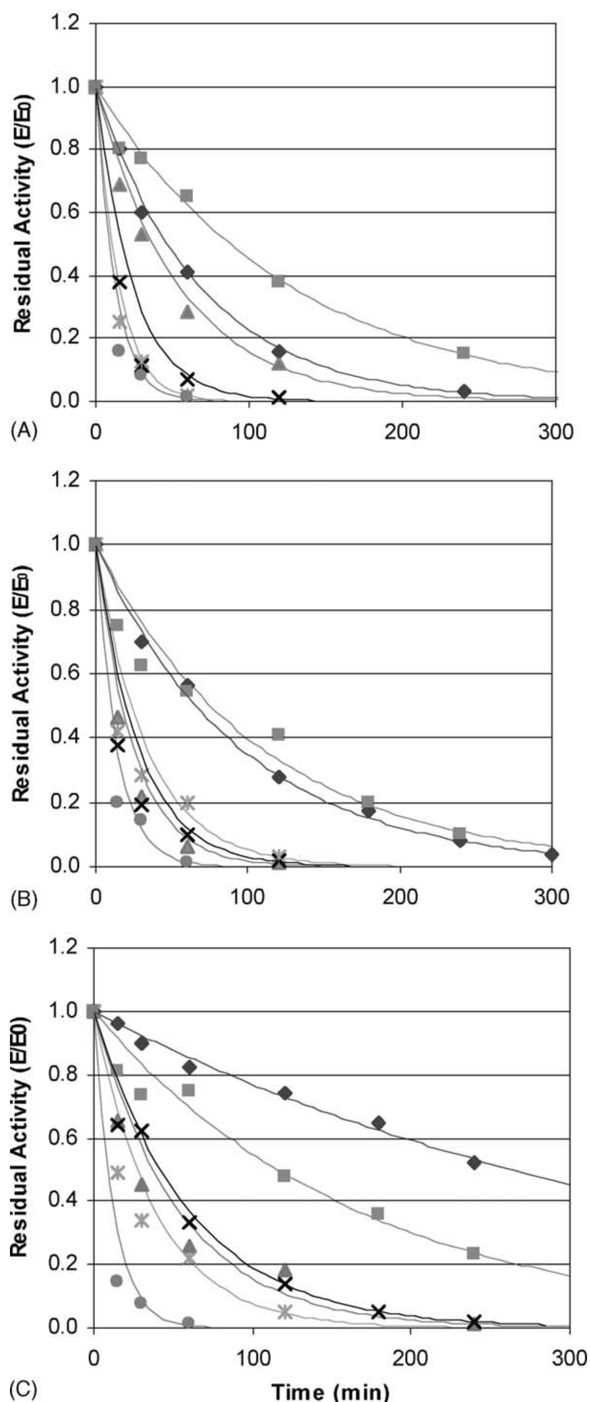

Fig. 1. Deactivation profiles of (A) free HRP, (B) free CBD–HRP, and (C) cellulose bound CBD–HRP in increasing concentrations of acetone–buffer mixtures (% acetone): (◆) 0%, (■) 20%, (▲) 40%, (×) 60%, (✳) 80%, and (●) 92%.

decrease in $t_{1/2}$ (Table 3). The exception is the higher stability of the free enzymes (HRP and CBD–HRP) at 20% acetone. The exact reason for the enhanced stability of HRP at low acetone concentrations is not clear. It has been reported for various enzymes that denaturation proceeded in a threshold manner in mixed single-phase aqueous organic solvent environments [41]. Thus, a variety or proteins were shown to retain complete biological function at low concentrations of polar solvents. Azevedo et al. observed that free HRP retained its stability in DMSO concentrations of 0–20% whereas the immobilized enzyme (covalently linked to silica microparticles) was more stable at 35% DMSO compared with the buffer. Gupta and co-workers who studied the behavior of HRP in various water organic solvent mixtures suggested that the presence of low levels of organic solvents may resemble the natural cellular micro-environment more closely than the pure aqueous medium [30]. It is, thus, not unlikely that at low acetone concentrations, interaction with the solvent enhances the stability of HRP.

The general concept of enhanced stability of an immobilized enzyme with respect to the free enzyme was confirmed in our studies. There have been reports that immobilization of enzymes by multi-point attachment protects them from denaturation by organic solvents in co-solvent mixtures [42,43]. Our immobilization method involves strong (nearly irreversible) binding of the CBD–HRP to cellulose beads via hydrophobic surface interactions of the CBD moiety. The major interaction is characterized by the planar strip of aromatic residues, which align along or across the cellulose chain [44,45]. Besides ensuring a stable immobilized complex, we believe that the CBD also acts as a mediator between the HRP molecules and the cellulose beads. It has been shown that one of the most important driving forces of protein passive adsorption onto matrices, is hydrophobic interaction. This interaction is thermodynamically favorable and, therefore, a protein will spread out hydrophobic residues in order to maximize this effect, leading to its inactivation [46]. In the case of CBD–HRP, the strong unidirectional binding of CBD to the cellulose dominates other interactions and, therefore, the HRP itself will remain intact. All of these considerations point to the achievement of a highly stable enzyme preparation.

Table 3
First-order inactivation constants (K_d), and half-life ($t_{1/2}$) of free and immobilized HRP in increasing concentrations of acetone

Acetone (%)	HRP		CBD–HRP		Cellulose–CBD–HRP	
	K_d (min^{-1})	$t_{1/2}$ (min)	K_d (min^{-1})	$t_{1/2}$ (min)	K_d (min^{-1})	$t_{1/2}$ (min)
0	14.8×10^{-3}	47	10.5×10^{-3}	66	2.5×10^{-3}	277
20	8.0×10^{-3}	87	9.2×10^{-3}	75	6.0×10^{-3}	115
40	18.4×10^{-3}	38	4.7×10^{-3}	15	18.4×10^{-3}	38
60	41.4×10^{-3}	16	35.1×10^{-3}	19	16.4×10^{-3}	42
80	67.1×10^{-3}	10	29.7×10^{-3}	23	25.7×10^{-3}	27
92	80.3×10^{-3}	8	72.0×10^{-3}	9	81.1×10^{-3}	8

Reaction conditions: 1 ml acetone–Tris buffer containing (a) 0.12 μg/ml HRP or (b) 8 μg/ml CBD–HRP or (c) 4 μg cellulose–CBD–HRP lyophilized from 10% sucrose solution. Solutions were incubated at 40 °C. Samples were removed periodically and residual activity was measured using the TMB substrate-kit.

3.4. Activity of HRP in aqueous-organic solvent mixtures

Enzymatic activity in organic solvents is often correlated with log *P*, the hydrophobicity index of the solvent. The higher the log *P* value, the more hydrophobic the solvent, and thus, the greater the enzymatic activity [47]. However, the log *P* value cannot be applied for aqueous mixtures containing water-miscible organic solvents [31,48,49]. We therefore chose, on the basis of published work regarding HRP, four commonly used solvents to examine the activity of our HRP derived preparations. The initial relative activity rates as a function of solvent concentration are presented in Fig. 2 (the initial activity in buffer was taken as 100%).

There was a strong dependence of solvent type on enzymatic activity. Acetonitrile, for example, caused rapid deterioration in activity compared with ethanol or acetone. In addition, there was a general decrease in activity with the rise in solvent concentration. However, in all solvents and at all concentrations the immobilized enzyme showed higher activity than the free enzymes. A pronounced effect was seen in 20% acetonitrile in which the free enzymes lost nearly all their activity, while the immobilized enzyme retained most of its activity. This means that immobilization onto cellulose via CBD prevented unfolding of the enzyme by the organic solvents. Immobilization of enzymes on supports is known to improve activity in organic media. One reason is that adsorption of the enzyme onto microporous matrices improves enzyme dispersion, reduces diffusional limitations and favors substrate access to individual enzyme molecules [34]. Another explanation is that the support provides a favorable microenvironment for the enzyme during catalysis, thereby increasing the catalytic activity [50].

In acetone, THF and ethanol, the immobilized enzyme was activated at 20% solvent concentration relative to its activity in buffer. On the one hand, the solvent system improves the solubility of the substrates and products but on the other hand, it promotes the unfolding of the native structure of the enzyme. In the case of cellulose-bound CBD–HRP the denaturation process is likely to be suppressed, and therefore, the solvent has a net positive influence on the activity.

Our results are in accordance with those of Azevedo et al. [31] who measured the enzymatic activity in increasing concentrations of DMSO, and found that covalently linked HRP to silica was more active than the free enzyme. Batra et al. [30] examined the effect of solvent mixtures on HRP immobilized by adsorption to chitosan and eudragit. Both free and chitosan-bound-HRP showed a 20% increase in activity in 10% acetonitrile and THF. This is similar to our results on immobilized HRP in THF, but not in acetonitrile. Another peroxidase enzyme, chloroperoxidase, was examined by Allain et al. in olefin epoxidation reactions [51]. They reported that addition of 25% acetone to the buffer medium improved the reactivity and the selectivity of the enzyme. The results in this study substantiate previous work regarding immobilization of HRP. The activity in co-solvent mixtures, as well as the stability, increases following immobilization of the enzyme.

It was interesting to note the differences between HRP and the fusion protein CBD–HRP, both in

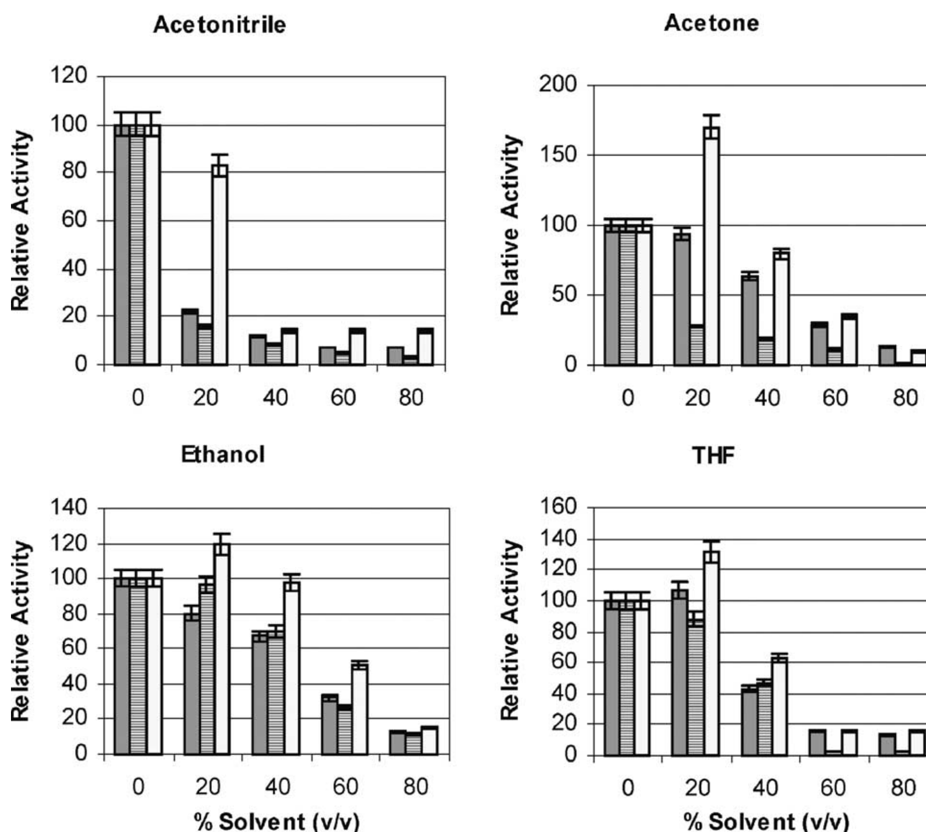

Fig. 2. Relative activity of free HRP (▦), CBD–HRP (▤) and cellulose–CBD–HRP (□) in solvent–buffer mixtures.

soluble form. In most of the solvents, the relative activity of CBD–HRP was lower than the relative activity of HRP, with increasing co-solvent concentrations. Even in the buffer alone a lower specific activity of CBD–HRP compared to that of HRP was measured (1 versus 17.9 μM/min mg, respectively). It is likely that the active site of the enzyme is sterically hindered as a result of the linked large structure. It was recently reported that in HRP, side-chain local reorganization, adjust the charge distribution of the protein matrix to allow aromatic substrate binding [52]. In other words, the enzyme modulates substrate binding by side chain reorganization rather then secondary structure conformational changes. Based on these findings, it is likely that in our study the presence of CBD interferes with the side chain reorganization when the CBD–HRP is in solution, whereas in the immobilized form this interference is reduced.

In contrast to the activity tests, the stability of CBD–HRP is higher than HRP in buffer and in high concentrations of acetone (Table 3). This may indicate a reduced degree of freedom to the unfolded state of CBD–HRP, which stabilizes the enzyme against denaturation, as does the immobilization onto a non-soluble support or chemical conjugation to modifiers such as polyethylene glycol (PEG) [53]. It is reported that modification of enzymes with covalently linked PEG enhances thermostability and stability in organic solvents, however, the activity is often markedly reduced [40,54]. It should be noted that reports claiming opposite observations, namely, reduction in stability of PEG-modified enzymes, were published [34]. Unlike chemical modification, which inherently involves modification of different amino acid side chains in random fashion, the genetically modified CBD-fusion enzyme results in a

homogeneous product. Thus, CBD-fusion enzymes could be, therefore, viewed as "genetically modified" enzymes, similar to PEG-enzymes in their increased stability accompanied by reduced activity, but with the advantage of homogeneity.

The advantages of the CBD–HRP fusion protein should be weighed against its reduced specific activity relative to that of HRP. Firstly, the level of expression of the fused protein was approximately five-fold higher than that of the native HRP. This compensates largely for the reduced specific activity (results not shown). Secondly, HRP was used only as a model enzyme and it is conceivable that with other enzymes the interference by fusion to CBD will be smaller. Furthermore, other types of CBDs may also give better results. Thirdly, the commercial employment of recombinant technology to immobilized enzymes via CBD onto cellulose has many advantages over chemical and physical methods of immobilization by virtue of its simplicity and cost effectiveness.

3.5. Thermostability of HRP

The denaturation profiles of HRP at various temperatures followed the exponential model described in the stability experiments. From these curves the half-life ($t_{1/2}$) of each enzyme form was calculated and could be used as a measure of its thermostability (Fig. 3).

The thermostability of both free and immobilized HRP decreased with the increase in temperature, however, the immobilized enzyme was more stable than the free form at all temperatures. At 40 °C, immobilized HRP was significantly more stable then HRP and CBD–HRP. At 50 °C, the differences between free and immobilized enzyme decreased and at 60 °C the trend reversed, i.e. soluble CBD–HRP was considerably more stable than the immobilized enzyme. At 70 °C, all enzymes lost their activity immediately (<10 min). The increased thermostability of the immobilized enzyme could be attributed to the rigidity of the enzyme structure following attachment to the support. One other example exists in which the thermal stability of cellulose bound CBD-β-glucosidase was reported to be higher than that of the native enzyme [20]. However, the thermal behavior of soluble CBD-fusion protein has not been studied to date.

From studies preformed on the inactivation mechanisms of hyperthermophilic proteins, the current hypothesis is that these enzymes are more rigid than their mesophilic homologues and that rigidity is a prerequisite for high protein thermostability [38]. It has also been proposed that high rigidity explains why hyperthermophilic enzymes are often inactive at low temperatures (20–37 °C). One set of evidence that supports this hypothesis is that denaturants like urea and solvents often activate hyperthermophilic enzymes at sub-optimal temperatures [55,56]. This

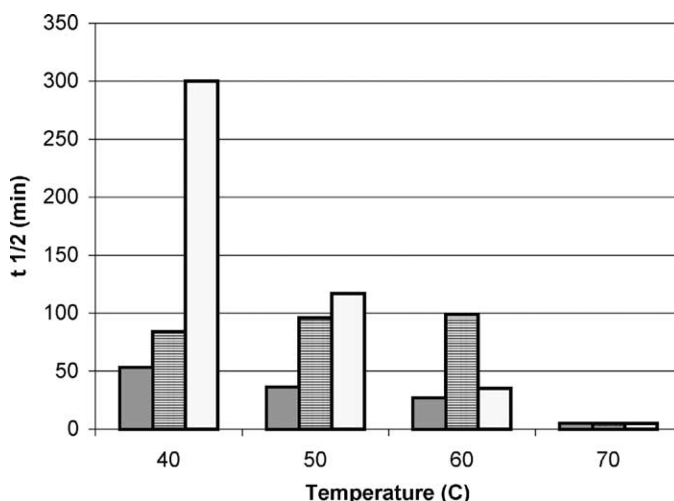

Fig. 3. Half-life of free HRP (▩), CBD–HRP (▤) and cellulose–CBD–HRP (□) at various temperatures, in buffer.

activation tends to disappear at temperatures closer to the enzyme's optimal activity where the enzyme is flexible enough in the absence of a denaturant to show full activity. Other reports have indicated that increased number of ionic and hydrogen bonding, and improved hydrophobic packing in the hydrophobic core of the enzyme are responsible for the increased rigidity [57,58].

The influence of CBDs on the thermostability of the naturally harboring enzymes (e.g. cellulases and xylanases) was studied and it was shown that deletion of these domains lead to decreased thermostability of the enzyme [59–61]. Furthermore, it was shown that the thermostability cannot be attributed solely to the CBD–cellulose interaction, but it involves an additional intra-molecular stabilizing interaction between the CBD and the enzyme [62]. In addition, binding of CBD to cellulose increases the thermal stability of CBD itself [63,64].

Considering the data presented above it is not surprising that CBD-fused–HRP exhibited thermostability when free in solution or when immobilized on cellulose. We suggest, that attachment of a bulky CBD molecule to HRP, increases its energy dissipation without denaturing. This effect is significant at temperatures up to 60 °C. At higher temperatures, CBD and HRP both undergo denaturation. It is possible that CBD originated from thermostable organisms will further improve the thermostability of enzymes immobilized on cellulose.

4. Conclusions

The work described in this paper demonstrates for the first time the use of a cellulose-bound CBD-fusion enzyme as a catalyst in aqueous-organic media. The CBD–HRP fusion protein containing two different functionalities, a catalytic domain and a binding domain, preserved both capabilities in the non-conventional environment. A six-fold increase in the half-life of the enzyme in buffer resulted from immobilization onto cellulose via CBD. The immobilized enzyme was also more stable in increasing concentrations of acetone (0–92%). There was a general decrease in activity with the rise in solvent concentration for all solvent types: THF, acetone, acetonitrile and ethanol. However, the immobilized enzyme was at all times more active than the free enzymes CBD–HRP and HRP. The cellulose bound enzyme exhibited higher activity in 20% acetone, ethanol and THF as compared with the buffer, followed by subsequent decline in higher solvent concentrations. This could be due to an overall positive balance between better solubilization of the substrate (and thus better contact with the enzyme) versus denaturation caused by the solvent. The thermostability of the enzyme in the temperature range of 40–60 °C was also improved following immobilization. It is important to note the greater stability (both to organic solvents and temperature) of the CBD–HRP fusion protein in comparison with the native HRP. It is believed that the more complex three-dimensional structure formed reduces the susceptibility to unfolding, similar to the stabilization achieved by modification of enzymes using PEG. The reduced activity of CBD–HRP is thought to be caused by steric hindrance. Work on other CBD-enzyme fusions is required in order to determine the generality of the method and to evaluate its commercial potential.

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