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Expression, Refolding and Indirect Immobilization of Horseradish Peroxidase (HRP) to Cellulose via a Phage-selected Peptide and Cellulose-binding Domain (CBD)

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Abstract: We examined the potential immobilization of horseradish peroxidase (HRP) to cellulose with cellulose-binding domain (CBD) as a mediator, using a ligand selected from a phage-displayed random peptide library. A 15-mer random peptide library was panned on cellulose-coated plates covered with CBD in order to find a peptide that binds to CBD in its bound form. The sequence I/LHS, which was found to be an efficient binder of CBD, was fused to a synthetic gene of HRP as an affinity tag. The tagged enzyme (tHRP) was then immobilized on microcrystalline cellulose coated with CBD, thereby demonstrating the indirect immobilization of a protein to cellulose via three amino acids selected by phage display library and CBD.

Keywords: immobilization; phage display library; horseradish peroxidase; cellulose-binding domain

INTRODUCTION

Random peptide libraries have been used to discover novel peptides with an affinity to molecular targets. Parmley and Smith [1] showed that foreign DNA fragments can be inserted into gene III of a filamentous phage to create an infective 'fusion phage' that displays foreign peptides on its surface. These peptides are accessible to other proteins and non-protein determinants, thereby allowing the isolation of phages that interact with specific determinants.

Protein immobilization to a solid phase is extensively used in ELISA [2], as well as in the concentration and purification of recombinant protein [3]. Conventional immobilization of proteins by direct passive adsorption to either plastic or other surfaces results in their partial or complete denaturation because only hydrophobic regions, which are usually inside the protein molecules, are able to interact with the polystyrene [4]. Affinity tags are commonly used and can be easily engineered either by the addition of a restricted fragment encoding the tail to the target protein gene or by insertion of the target protein gene into a vector that contains the tail [3]. Different methods have been developed to avoid protein denaturation during coating, such as the streptavidin–biotin specific interaction [5] or the albumin-binding protein domain (ABD), which interacts with high affinity to rat serum albumin (RSA) [6]. These systems usually require the use of expensive reagents or the fusion of large proteins to

Abbreviations: ABD, albumin-binding protein domain; CBD, cellulose-binding domain; HRP, horseradish peroxidase; IPTG, isopropyl-D-thiogalactopyranoside; pfu, plaque-forming units; RSA, rat serum albumin; TMB, 3,3′,5,5′-tetramethylbenzidine.

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the target proteins. A small tag is often seen as being advantageous for several reasons: (a) it is less likely to affect interaction of the target protein with its substrate; (b) it may be less antigenic; and (c) from a production point of view, the expression of a large tag is a waste of resources. An example of a small tag is poly-His, which is commercially available (Novagen, Madison, WI) [7]. Although very popular, the poly-His tag suffers from two major disadvantages: (a) the relatively high, non-specific binding of other metal-binding proteins to the solid phase; and (b) it is very hydrophobic and is often buried in the fusion partner, thus eliminating the possibility of binding the target protein in its native fold to the solid phase.

Cellulose-binding domains (CBDs) are found in nature as discrete domains in proteins such as cellulases [8], as well as in proteins with no hydrolytic activity [9]. In cellulases, it is thought that CBDs concentrate their catalytic domains on the surface of the insoluble cellulose substrate. In proteins with no hydrolytic activity, the CBD is part of a scaffolding subunit that organizes the catalytic subunits into a cohesive, multienzyme complex known as a cellulosome [10]. The *Clostridium cellulovorans* scaffolding gene (*cbpA*) was cloned and sequenced [11], and its CBD was successfully expressed in *Escherichia coli*. The recombinant protein exhibits high affinity to cellulose [12] and is used to immobilize different proteins to cellulose [13–15]. Nevertheless, the CBD derived from *cbpA* is a 17-kDa protein and thus falls into the category of relatively large tags.

In the present study, we investigate the possibility of using a small peptide tag (four amino acids) to immobilize a protein to a solid surface via a non-covalent interaction with a cellulose-binding protein (illustrated in Figure 1). This technique may enable future investigators to tailor-make fusion tags that will mediate non-covalent binding to solid phase matrices.

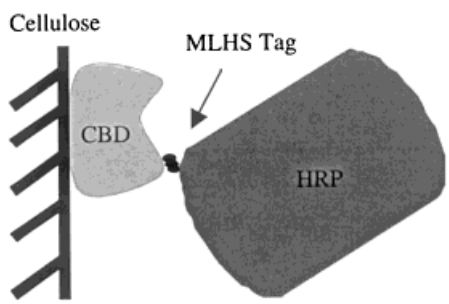

Figure 1 Schematic presentation of the working model.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli*

XL1-Blue was used for all experiments with phage M13. *Escherichia coli* DH5α (Life Technologies Inc., Rockville, MD, USA) was used in all of the cloning procedures. *Escherichia coli* BL21 (DE3) was used for expression using the vector pET29a(+) (Novagen Inc., Madison, WI, USA).

Enzymes and Chemicals

Chemicals were purchased from Sigma Israel Chemicals Ltd. (Rehovot, Israel) unless stated otherwise. Enzymes were purchased from MBI Fermentas (Vilnius, Lithuania). *Taq* DNA polymerase was purchased from Promega Corp. (Madison, WI, USA). CBD_{Clos} was kindly provided by CBD-Technologies Ltd. (Rehovot, Israel).

DNA Manipulation

Standard DNA manipulations and PCR amplification procedures were based on the work of Sambrook *et al.* [16] and Scharf [17], respectively.

Phage Display Library

The 15-mer M13 phage peptide library was provided by Devlin [18]. The library consists of 2.8×10^7 different 15-amino acid *N*-terminal fusion in gIIIp.

Cellulose Acetate-coated Plates

Plates were coated with cellulose acetate as described by Wierzba *et al.* [19], with the following changes: cellulose acetate was dissolved in glacial acetic acid at 1% (w/v); 1 ml of the solution was placed in each well of a 24-well untreated, polystyrene plate (Nunclon, Nunc, Denmark). Plates were turned upside down in the fume hood overnight. The resultant coating was transparent with no apparent optical interference.

Screening for CBD Binding Peptide

A sample of the phage library was subjected to three rounds of selection (panning) and amplification. Each round consisted of the following procedure: applying the sample library (10^{10} plaque-forming units (pfu) in the first round, 10^7 in the second round and 10^6 in the third round), in the presence of 990 μl 20 mM citrate phosphate buffer pH 6.0 for 1 h, to a 24-well cellulose acetate-coated plate. The sample was then transferred to cellulose-coated

wells that had previously been incubated with 200 μg/ml CBD for 1 h in 20 mM Tris base pH 7.0, and was incubated for another hour. After three washes with 1 ml 20 mM citrate phosphate buffer pH 6.0, the phages were eluted with 1 ml 20 mM glycine buffer pH 9.0 for 10 min. The eluted phages were amplified as described by Adey *et al.* [20]. After the three rounds, 50 plaques were randomly picked and each clone was mixed with wild-type M13mp19 in order to find the relative enrichments of the peptide-bearing phage ('white enrichment test') as described by Devlin *et al.* [18].

Construction of pET29a-HRP and pET29a-tHRP

The kanamycin-resistant *E. coli* expression vector pET29a(+) was used to express the *hrp* and t*hrp* genes. The synthetic HRP gene, EC 1.11.1.7 (R&D Systems Inc., Minneapolis, MN, USA) containing *Nde*I/*Bam*HI restriction sites, was cloned directly into the pET29a(+) vector, resulting in pET29a-HRP. In order to fuse the tag sequence, we designed a 5′ primer (pr5′: 5′-AAAACATATGCTGCACAGCG-CGCCCATGCAATTAACCCCTACATCCTAC-3′) containing the *Nde*I site upstream of the sequence MLHSAP (using *E. coli* codon usage). Ala and Pro were used as connecting amino acids between the tag sequence and the *hrp* sequence. The 3′ primer (pr3′: 5′-GAATTCGGATCCTTATTAAGAGTTGC-3′) contained the *Bam*HI restriction site. The HRP coding sequence was PCR-amplified (Rapidcycler, Idaho Technology Inc, Idaho Falls, ID, USA) using pr5′ and pr3′ primers (94°C for 1 min, 30 cycles of 94°C for 10 s, 55°C for 10 s, 72°C for 35 s, and 72°C for 5 min) and the synthetic *hrp* gene as a template. The PCR product, which now contained the tag sequence, was digested with *Nde*I/*Bam*HI and cloned into the pET29a(+) which had been predigested with the same enzymes resulting in pET29a-tHRP. The ligation mixture was used to transform *E. coli* DH5α and the presence of the tag sequence was confirmed by sequencing.

Expression of HRP/tHRP in *E. coli*

HRP and tHRP were overexpressed in *E. coli* BL21 (DE3) harbouring the pET29a-HRP or pET29a-tHRP plasmids. Inoculum was prepared by growing a single colony in 4 ml LB (10 g/l bactotrypton, 5 g/l yeast extract, 10 g/l NaCl) medium containing 50 μg/ml kanamycin at 37°C to an OD_{600} of 0.6–1.0. After adding 2 ml inoculum to 100 ml fresh LB medium containing 50 μg/ml kanamycin, cells were grown in shaking flasks at 250 rpm and 37°C to an OD_{600} of 0.6, after which 1 mM (final concentration) isopropyl-D-thiogalactopyranoside (IPTG) was added. Following 4 h of incubation, the cells were harvested by centrifugation at 1600 *g* for 10 min, 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, 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 15000 *g* for 10 min followed by four washes in 20 ml 20 mM Tris pH 8.0 containing 1 mM EDTA and 1% Triton.

Refolding of HRP

Inclusion bodies were dissolved in urea solution (4.5 M urea, 40 mM Tris base pH 11.3 and 1 mM Cys) to a protein concentration of 0.1 mg/ml, as determined by bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), and stirred for 1 h to solubilize the inclusion bodies. The denatured proteins were dialysed for 5 h at 4°C against 20 mM Tris base pH 8.5 containing 0.5 M urea, 0.2 mM hemin, 5 mM $CaCl_2$ and 150 μM oxidized glutathione (first dialysis), and then for 5 h at 4°C against 20 mM Tris base pH 8.5 in 50% (v/v) glycerol containing 5 mM $CaCl_2$ (second dialysis). The protein solution was analysed by 12.5% SDS-PAGE [21]. Specific activity was determined according to Josephy *et al.* [22] using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate.

Binding of HRP/tHRP to CBD

One microgram of microcrystalline cellulose (20 μm average size; Sigmacell, Sigma Chemicals Ltd., Rehovot, Israel) was mixed with 200 μg CBD in 0.5 ml 20 mM Tris base pH 8.0 and was incubated with constant rotation at room temperature (RT) for 1 h. The cellulose was centrifuged at 16000 *g* for 1 min and unbound CBD was washed away with 1 M NaCl followed by two washes with 0.5 ml 20 mM Tris base pH 8.0. Then, 20 μg HRP was added to the cellulose, or to cellulose precoated with CBD, in 0.5 ml 20 mM citrate phosphate buffer pH 6.0, and incubated with constant rotation at RT for 1 h. The cellulose was centrifuged at 16000 *g* for 1 min and unbound HRP was removed with five washes in 0.5 ml of 20 mM citrate phosphate buffer pH 6.0. To quantify the amount of bound HRP, we added 200 μl TMB (TMB substrate kit, Pierce) and incubated the tubes for 30 min at RT with constant rotation. The tubes were then centrifuged at 16000 *g* for 1 min and the

supernatant was transferred to 96-well ELISA plates (Nunclon). The OD_{655} was determined using an ELISA reader (Microplate Manager III, Bio Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analyses were performed using JMP 3.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

The 15-mer library was subjected to three rounds of affinity selection against the CBD molecule immobilized on a cellulose matrix. Screening was performed in two steps: first on a plastic well coated with cellulose without CBD, in order to subtract background phages that interact with either plastic or cellulose, and then on a cellulose well coated with CBD. In order to maximize the positive clones, we selected 50 plaques after the third panning round and applied the white enrichment test [18]. The enrichment data in Table 1 indicate that the maximum enrichment occurred in the first panning round. Thirty plaques that showed more than a 10-fold enrichment were sequenced and their DNA analysis predicted different random peptide sequences, with almost all appearing more than once (Table 2). The aligned sequences illustrated in Table 2 indicate an I/LHS consensus sequence that appeared in 14 out of 30 clones sequenced. His was found in 23 clones, in the context of either I/LHS, LH or HS consensus sequences.

All amino acids were represented, although their frequencies did not always correspond to that expected (Figure 2). However, some residues, such as His, Tyr, Lys and Pro, tended to be more abundant, whereas the reverse was true for others (Arg, Gly, Trp and Val) (Figure 2). Most sequences (23 out of 30) showed a repeating pattern of hydrophobic (Ala, Ile, Leu and Thr), positively charged amino acid side chains (His) and hydrophilic, uncharged amino acid side chains (Ser, Gln, Tyr and Lys).

The overall structure of the pET29a-HRP vector is shown in Figure 3. Using the synthetic *hrp* gene as a template for PCR, we cloned the t*hrp* gene to include the tag sequence at the *N*-terminal end of HRP. The *hrp* gene was cloned directly into the pET vector, without the use of PCR. The integrity of the DNA sequence of both clones was determined by sequencing. HRP was overexpressed in *E. coli* BL21 (DE3) cells harbouring the pET29a-HRP or pET29a-tHRP expression vector. Total *E. coli* proteins were analysed by SDS-PAGE (Figure 4). HRP and tHRP were expressed at high levels (at about 34 kDa), and accumulated as inclusion bodies similar to what was previously reported by Smith *et al.* [23] and Doyle and Smith [24]. Very low peroxidase activity was found in the soluble fraction, but no activity was found in the inclusion bodies before refolding (data not shown).

A simple refolding protocol, revised from Smith *et al.* [23], was conducted. The procedure included a solubilization step at high pH in the presence of 4.5 M urea, a refolding step in the presence of oxidized glutathione and a concentration step with glycerol. This procedure resulted in a high HRP concentration (Table 3). The specific activity was found to be 4.16 units/μg for tHRP and 5.83 units/μg for HRP, as determined according to Josephy *et al.* [22] using TMB as the substrate. Our refolding procedure yielded relatively high levels of recovery and purity (Figure 4).

Table 1 Results of Three Cycles of Biopanning against CBD

Rounds of panning	No. of phages applied (pfu/ml)	No. of phages eluted (pfu/ml)	Percent recovery (%)
1	3.1×10^{10}	4×10^{3}	0.000013
2	2.4×10^{7}	3.5×10^{4}	0.146
3	8.3×10^{6}	2.4×10^{6}	29

pfu = plaque-forming unit.
Fixed amounts of phages were biopanned on cellulose-coated polystyrene plates pretreated with CBD (200 μg/ml). After three washes, the phages were eluted and plated as described by Adey *et al.* [20]. Plaques were then counted.

Table 2 Analysis of the Predicted Amino Acid Sequences from Selected Clones

Clone	Peptide sequence	Frequency
1	N A V A **H** Q A V G P A P F L S	3
2	P Q H Y K M P V R P Y S **I H S**	4
3	L P Y S H K F M **I H S** K P T F	6
4	S S T R S L T F D F N M **L H S**	4
5	M D A Q H L V A **L H** D V A F Y	4
6	P T P C H G Q V D E **L H** A A V	1
7	W T P S T L S **H** Y M T S P F Y	2
8	S C C T **H S** T P A L P Q L P S	4
9	H L L T **H** K A H D N A Y Y A K	2

After the third round of affinity selection against CBD followed by the 'white enrichment test' [17], the nucleotide sequences of 30 recovered phages were analysed and their amino acid sequences determined. The sequences, given in single-letter code, were aligned, yielding an I/LHS consensus sequence. The number of times each sequence occurred is given in the frequency column.

CBD was bound to microcrystalline cellulose and excess CBD was washed away before binding to HRP or tHRP. The ability of tHRP to bind to cellulose was estimated by measuring the enzymatic activity of the cellulose matrix. tHRP could be eluted from the CBD-coated cellulose beads by 20 mM citrate phosphate buffer pH 3. Under these conditions, CBD remains bound to the cellulose. Several controls were tested, including cellulose without CBD and HRP without the tag. Although non-specific binding to cellulose and CBD-coated cellulose was observed for HRP, tHRP bound significantly more to CBD-coated cellulose but not to non-coated cellulose (Figure 5). ELISA of tHRP bound to a CBD-coated polystyrene plate versus a BSA-coated plate indicated that the peptide tag specifically binds to CBD but not to BSA (data not shown).

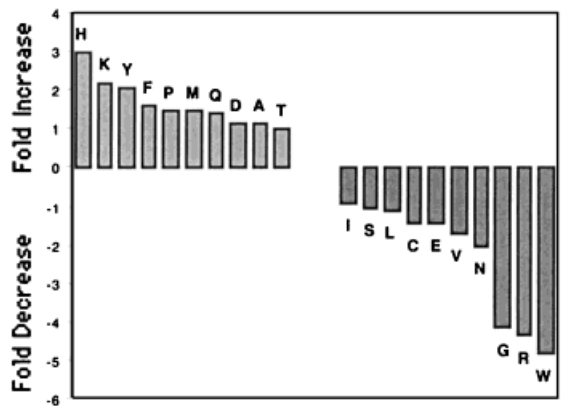

Figure 2 Relative abundance of 20 amino acids in the 15-mer library after selection for CBD binding. The overall amino acid composition of all 30 clones was compared with the calculated amino acid composition. In this library, only 32 codons (out of a potential 64) encode all of the amino acids. Cys, Asp, Glu, Phe, His, Ile, Lys, Met, Asn, Gln, Trp and Tyr are encoded by one codon; Ala, Gly, Pro, Thr and Val are encoded by two codons; and Leu, Arg and Ser are encoded by three codons. The calculated number of appearances was calculated by multiplying the relative number of codons that encode each amino acid by the total of 450 amino acids present in the 30 clones sequenced. The fold increase was calculated by dividing the total number of appearances by the calculated number of appearances, and vice versa for fold decrease.

DISCUSSION

Previous studies have shown that recombinant libraries displaying short random peptides can be a valuable resource for identifying peptide sequences that have an affinity to various targets. These libraries have yielded peptides that resemble antigen epitopes [21,25], as well as peptides that mimic non-peptides [18]. Phage display systems usually involve an obligatory display of peptides on the phage surface, potentially allowing selection and enrichment based on avidity and affinity. In our work, subsequent analysis of the enriched peptides revealed the sequence Ile/Leu, His, Ser in the consensus pattern of hydrophobic, positively charged and hydrophilic amino acids.

A distribution bias of peptides in the library may be caused by either biological selection or structural constraints within the peptides. Various studies have reported that the library bias usually favours Gly and disfavours Pro and Cys [26–30]. In our study, we show the opposite: a strong, negative

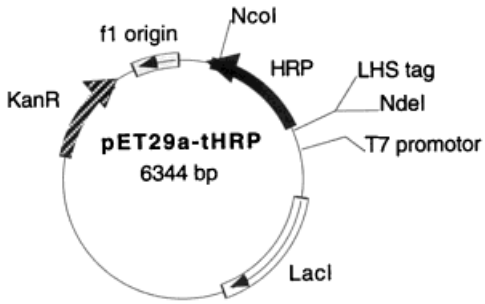

Figure 3 Schematic diagram of the pET29a-tHRP vector. The map of the pET29a-tHRP vector shows the relative position of the HRP gene as well as the tag sequence position. The LHS peptide was cloned at the 5′ end of the HRP gene in the pET29a-tHRP vector, whereas the vector pET29a-HRP contains only the *hrp* gene.

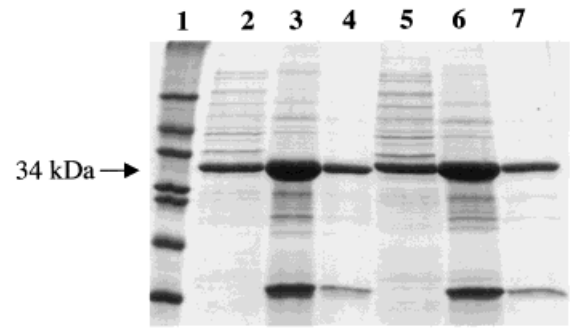

Figure 4 SDS-PAGE analysis of recombinant HRP and tHRP expression in *E. coli*. A 12.5% SDS-PAGE of various stages in the expression, purification and refolding of the 34-kDa HRP (lanes 2–4) and tHRP (lane 5–7). Lane 1: molecular weight standards (top to bottom: 66, 45, 36, 29, 24, 20.1 and 14.2 kDa). Lanes 2 and 5: total protein after induction with 1 mM IPTG. Lanes 3 and 6: purified inclusion bodies. Lanes 4 and 7: protein after refolding.

selection towards Gly and a weak, positive selection towards Pro. Looking at the relative abundance of the amino acids in the selected clones (Figure 2), one cannot find a consistent pattern in the means of positive/negative charge or hydrophilicity/hydrophobicity (in particular, differences between His and Lys versus Arg). These results imply the relatively high importance of the 'space-filling' function of the different amino acids.

When HRP and tHRP were overexpressed in *E. coli*, the proteins were produced in large quantities, approximately 50–60 mg protein/l (Table 3). The advantage of our method of expression and refolding over other previously described methods for HRP [23,24] lies in quantities, recovery percentage and ease of protocol.

The binding specificity of tHRP to CBD bound to cellulose was determined (Figure 5) and was found to be CBD-dependent, whereas HRP binding was not.

In this study, we selected a peptide from a phage display library that enabled the binding of an enzyme to solid cellulose via CBD as a mediator using only a three-amino acid tag. The feasibility of creating a very small tag that enables the immobilization of a target protein has been demonstrated. Given the large diversity of products and applications that make use of cellulose materials, this novel approach is expected to pave the way for many novel applications.

Table 3 Steps in the Expression and Purification of HRP and tHRP from Inclusion Bodies

	HRP/tHRP			
	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Percent recovery (%)
Fermentation broth	100/100	ND	ND	ND
Pure inclusion bodies	50/50	0.13/0.10	6.5/5.1	100/100
After the first dialysis	50/50	0.07/0.07	3.50/3.85	53/77
After the second dialysis (against glycerol)	15/15	0.09/0.13	1.40/1.97	21/39

ND: not determined.
HRP and tHRP were overexpressed in *E. coli*. Inclusion bodies were collected by lysing the bacterial cells and centrifugation. The refolding procedure consisted of three steps: dissolving inclusion bodies in buffer containing urea; dialysing against 20 mM Tris base pH 8.5 containing 0.5 M urea, 0.2 mM hemin, 5 mM $CaCl_2$ and 150 μM oxidized glutathione (first dialysis) and then against 20 mM Tris base pH 8.5 in 50% glycerol containing 5 mM $CaCl_2$ (second dialysis).

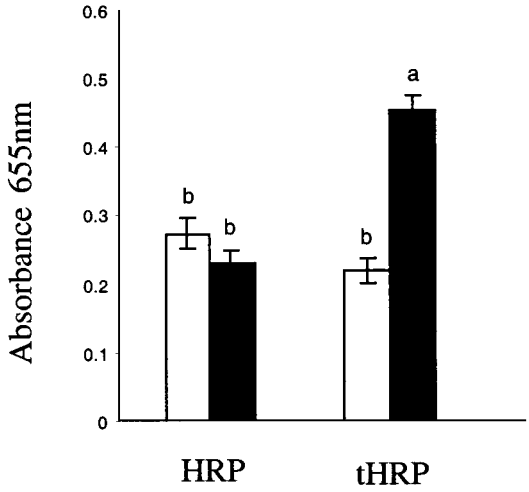

Figure 5 Immobilization of tagged HRP versus non-tagged HRP in the presence and absence of CBD. HRP and tHRP were applied to CBD-coated cellulose beads (black bars) and non-coated beads (open bars). The enzymatic activity represents the amount of immobilized protein. One-way ANOVA was conducted using pairwise comparisons by the Tukey–Kramer method ($p = 0.01$).

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