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Enhancing the Enzymatic Activity of a Heme-Dependent Peroxidase through Genetic Modification

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Academic Editors: David D. Boehr and Keith Hohn

Received: 28 August 2016; Accepted: 18 October 2016; Published: 27 October 2016

Abstract: A heme-dependent peroxidase (HDP) catalyzes the ortho-hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) in the presence of hydrogen peroxide. L-DOPA can be used for the treatment of Parkinson's disease. In this work, to improve the catalytic efficiency, the heme-dependent peroxidase has been genetically modified with an elastin-like polypeptide (ELP). bicinchoninic acid (BCA) assay demonstrated that HDP-ELP has a higher solubility in aqueous solutions than HDP. Circular dichroism (CD) spectra showed that HDP-ELP has a higher stability than HDP. Enzyme kinetics has been investigated over a range of substrate concentrations. It has been demonstrated that HDP-ELP exhibited a catalytic efficiency 2.4 times that of HDP.

Keywords: heme-dependent peroxidase; elastin-like polypeptide; hydrogen peroxide; solubility; stability

1. Introduction

Enzymes have many applications in biotechnology industries [1,2]. Enzyme immobilization can facilitate increasing enzyme stability, improving catalysis efficiency, and reducing the chance of contamination in products [3–6]. Various materials have been used to immobilize enzymes. Natural polymers have been extensively studied as supports for immobilization of enzymes/proteins, such as starch [7], alginate [8–10], chitosan [11,12], cellulose [13–15], and carrageenan [16]. Each natural polymer has its own characteristics. Alginate and starch have a good water holding capacity. Chitosan has hydroxyl and amino groups, which can be used to form covalent bonds with enzymes. Carrageenan can have a high protein holding capacity. Cellulose is abundant and relatively cheap.

Hydroxylation of tyrosine is an important reaction for synthesis of natural chemicals. The hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) can be catalyzed by a heme-dependent peroxidase in the presence of hydrogen peroxide [17]. L-DOPA is the precursor to synthesize neurotransmitters dopamine, norepinephrine and epinephrine.

Elastin-like polypeptides (ELPs) are a kind of artificial polypeptides [18]. With ELPs as tags, proteins and enzymes can be purified through reversible phase transition, and enzyme purification process can be simplified [19].In addition, ELPs can self-assemble to form nano-size aggregates [20]. Herein, an elastin-like polypeptide (ELP) has been fused to a heme-dependent peroxidase (HDP) through genetic engineering technology. The enzyme is linked to ELP with peptide bonds. The fused enzyme was expressed in *Escherichia coli*. Thus, immobilization of the enzyme occurred naturally in *Escherichia coli* cells, with little effect on the secondary structures of the enzyme. The fused enzyme has been purified through phase transition. During the enzyme purification process, only NaCl solution is utilized, it is not necessary to use chromatography column and adsorbents and other chemicals. The enzymatic activity of the fused heme-dependent peroxidase has been investigated.

2. Results and Discussion

2.1. Expression of Recombinant Heme-Dependent Peroxidase

The genes encoding an ELP and the HDP were joined through recombination recombination technology. *Escherichia coli* was the host strain for expressing the recombinant enzyme HDP-ELP. The expressed HDP-ELP was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), the band indicated by the arrow is for HDP-ELP (Figure 1a). It is indicated that HDP-ELP is approximately 68 kDa, which is consistent with the molecular weight that is predicted theoretically. With ELPs as tags, proteins and enzymes can be purified through reversible phase transition, and the purification process can be simplified [19]. According to the method in the article [18], the expressed HDP-ELP was purified with a high purity, and the contaminant proteins were significantly reduced (Figure 1a). The purified HDP-ELP was further analyzed by Western Blot. Figure 1b clearly shows two bands, lane 1 is for the heme-dependent peroxidase, and lane 2 is for the fused enzyme HDP-ELP.

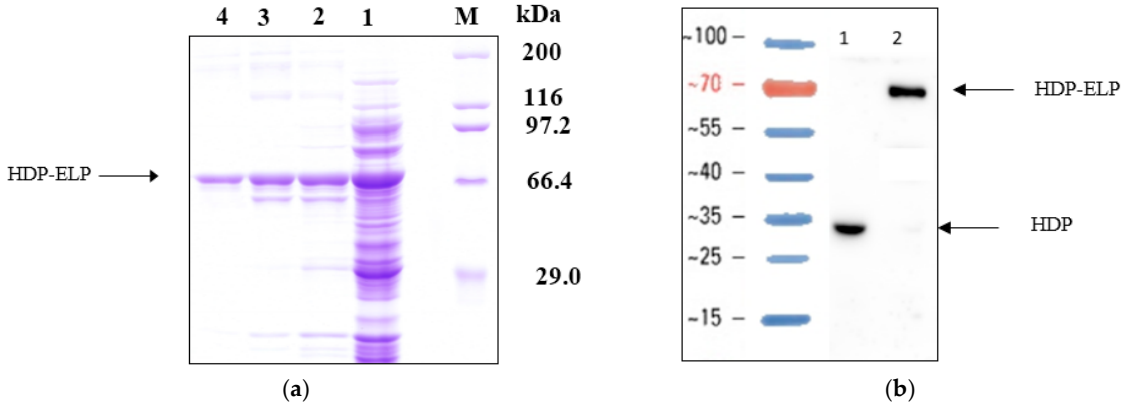

Figure 1. Analysis of purified heme-dependent peroxidase (HDP)-elastin-like polypeptide (ELP) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (**a**) and Western Blot (**b**). In (**a**), lane M is for Mark proteins (KDa); lane 1 is for all proteins expressed; lanes 2, 3 and 4 are for the purified HDP-ELP after first, secondary, and third round purifications, respectively. In (**b**), lane 1 is for HDP and lane 2 for HDP-ELP.

2.2. Improvement of HDP Solubility after Being Fused to ELP

A higher solubility of an enzyme in aqueous solutions means that the enzyme is well dissolved in the solutions, promoting the interaction of the enzyme molecules with substrate molecules. The solubility of HDP-ELP and HDP has been measured. Figure 2 shows the normalized solubility of the enzymes with incubation time. The solubility of the enzymes decreased with incubation time. After 90 min, HDP-ELP retained 93% of its initial solubility, while HDP only retained 63% of its initial solubility. In a separate test, both HDP and HDP-ELP have been used to catalyze the reactions. In the solution containing HDP-ELP (Figure 3), enzyme aggregation was not obviously observed after 90 min. In contrast, in the solution containing HDP (Figure 3), enzyme aggregated and precipitated onto the bottom of the tube after 90 min. The results in Figures 2 and 3 demonstrated that HDP-ELP has a higher solubility in aqueous solutions than HDP. A higher solubility is favorable for HDP-ELP to interact with the substrate L-tyrosine, and hence enhance the enzymatic activity.

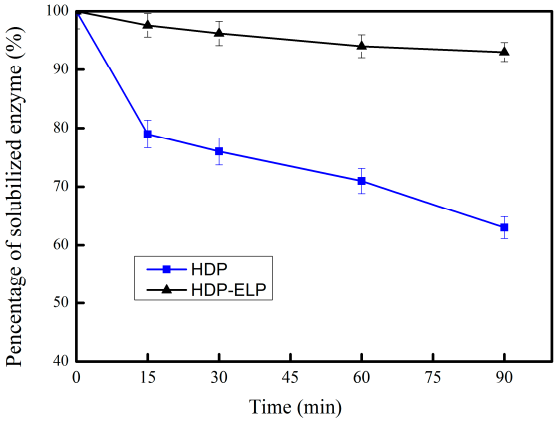

Figure 2. Comparison of enzyme solubility in aqueous solution.

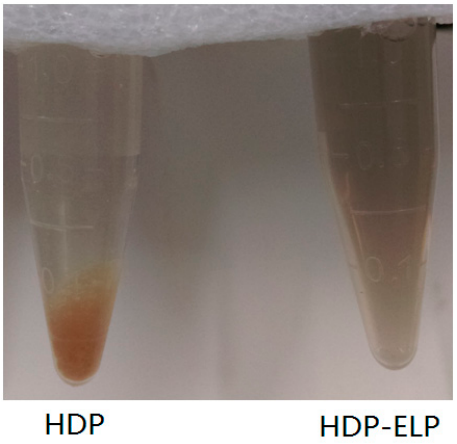

Figure 3. Solutions containing HDP and HDP-ELP after 90 min. In the tube containing the enzyme HDP, enzyme aggregated and precipitated onto the bottom of the tube after 90 min of reaction; in the tube containing HDP-ELP, enzyme aggregation was not obviously observed after 90 min.

2.3. Circular Dichroism (CD) Spectra at Different H_2O_2 Concentrations

For the ortho-hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) under the catalysis of HDP, hydrogen peroxide is required. However, hydrogen peroxide has been found to inactivate the enzyme during catalysis [17] and has a deleterious effect on enzymes [21].It is possible that hydrogen peroxide may interfere the secondary structures of the enzymes during catalysis. Herein the change of secondary structures of the enzymes in the presence of H_2O_2 with different concentrations was monitored by measuring CD spectra. To ensure a reliable measurement of CD spectra, the high tension voltage was limited to be less than 700 V [22]. Figure 4a,b show the CD spectra for HDP and HDP-ELP, respectively. Characteristic CD band shapes are attributed to protein secondary structures [23]. In the absence of H_2O_2 (control), HDP exhibited a typical CD pattern associated with secondary structures (Figure 4a). Upon increase of the concentration of H_2O_2, the spectral shape changes. At 5 mM, HDP exhibited a dramatically different spectral line shape, arising from the interfering effect of H_2O_2 on the secondary structures. In contrast for HDP-ELP (Figure 4b), in the H_2O_2 concentration range from 0 to 5 mM, the change of CD spectral shapes is very small, implying a smaller interfering effect of H_2O_2 on the secondary structures. The CD spectra demonstrated that stability of the heme-dependent peroxidase has been improved after being fused to the elastin-like polypeptide. Preserving the secondary structures of the enzyme against H_2O_2 interfering is essential to maintain its function as an efficient catalyst.

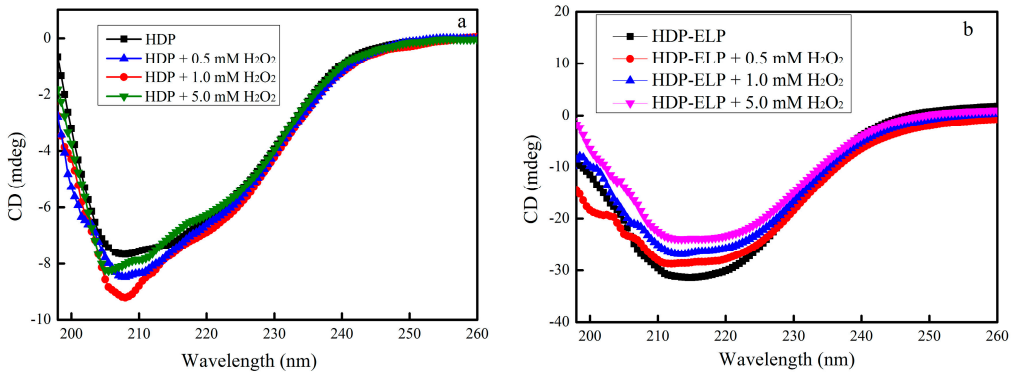

Figure 4. Effect of H_2O_2 concentration on the secondary structures monitored by circular dichroism spectra. (**a**) HDP; (**b**) HDP-ELP.

2.4. Improvement of Catalytic Efficiency

The heme-dependent peroxidase (HDP) catalyzed the ortho-hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA)in the presence of hydrogen peroxide [17]. Here both HDP and HDP-ELP have been used to catalyze the ortho-hydroxylation of L-tyrosine with various hydrogen peroxide concentrations from 0.5 to 3 mM. At the concentration of 1.0 mM H_2O_2, the L-tyrosine conversion reached the highest values, they were 21.1% (Figure 5a) and 70.3% (Figure 5b) for HDP and HDP-ELP, respectively. Further increasing the concentration of H_2O_2 did not promote the L-tyrosine conversions. The conversion results demonstrated that HDL-ELP has significantly improved the catalytic efficiency. Enzyme kinetics has been investigated over a range of substrate concentrations. The supplementary Figure S1 shows plot of the initial rate of reaction (V_0) and kinetics parameters were obtained. HDP-ELP exhibited a k_m value 0.57 ± 0.05 mM, in comparison to the k_m value 0.45 ± 0.04 mM for HDP [17]. It is indicated that HDP-ELP and HDP have a comparable affinity towards substrate. k_{cat} is the turn number. HDP-ELP exhibited a k_{cat} value 1.66 ± 0.04 s^{-1}, which is much larger than that of HDP 0.576 ± 0.005 s^{-1} [17]. The k_{cat}/k_m ratio has been used to measure catalytic efficiency [20]. The k_{cat}/k_m ratio of HDP-ELP (2.89 $mM^{-1}{\cdot}s^{-1}$) is 2.4 times that of HDP (1.2 $mM^{-1}{\cdot}s^{-1}$), indicating that a much higher catalytic efficiency has been achieved by HDP-ELP.As demonstrated in the above sections, HDP-ELP has a higher solubility in aqueous solution and has a higher stability against H_2O_2 interfering the secondary structures. These two aspects contribute to the improvement of catalytic efficiency. Furthermore, at different temperatures and pH conditions, the activity assay for HDP-ELP has been performed. The supplementary Figure S2 shows that in the temperature range from 20 to 40 °C and at the pH conditions from 7 to 10, HDP-ELP exhibited a stable enzymatic activity.

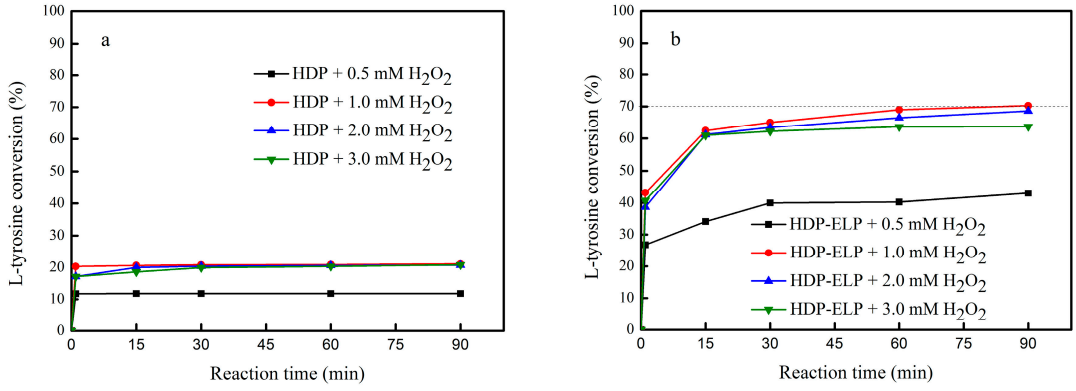

Figure 5. L-Tyrosine conversion by HDP and HDP-ELP with various concentrations of H_2O_2. (**a**) By HDP catalysis; (**b**) by HDP-ELP catalysis. The reaction temperature is 30 °C.

3. Experimental Section

3.1. Materials

Restriction enzymes, DNA polymerase and DNA ligase were obtained from New England Biolabs (Shanghai, China) and Fermentas (Burlington, Ontario, Canada). The gene for the ELP monomer is listed in Table S1. The heme-dependent peroxidase gene was synthesized by BGI Tech (Shenzhen, China) (Table S2), which is in accordance with the gene sequence in the article [17]. All other chemicals and reagents were obtained from Sinopharm Chemical Reagent (Shanghai, China) and Sigma-Aldrich (Shanghai, China).

3.2. Expression Vector Construction

The construction of the vector pET-28a-ELP has been described in supplementary materials, in which the gene sequence of the heme-dependent peroxidase is also listed. The synthesized HDP gene was in the pBM19-B vector. The extracted pBM19-B vector was digested with both the BamhI and SacI restriction endonucleases. The resulted HDP gene fragment was ligated into the vector pET-28a-ELP, the vector has already been digested with both the BamhI and SacI restriction endonucleases. Thus the expression vector pET-28a-HDP-ELP in *Escherichia coli* was constructed, as illustrated in Figure 6.

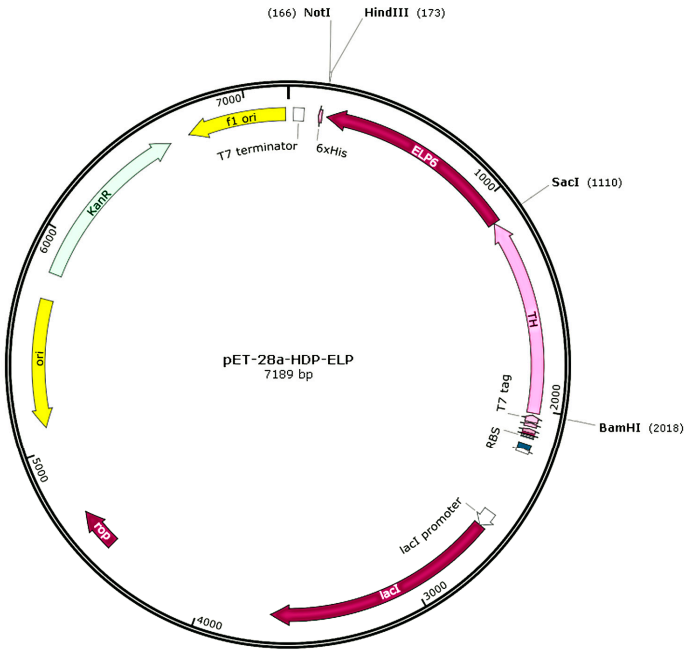

Figure 6. Schematic presentation of constructed vector for expression of HDP-ELP.

3.3. Enzyme Expression and Purification

The HDP was fused to an ELP. For expressing HDP-ELP, the vector was transferred to *Escherichia coli* BL21 cells, which were grown in 1 L of Luria–Bertani brothmedium at 37 °C. After the culture reaching an optical density at 600 nm of 0.6, isopropy-β-D-thiogalactoside (IPTG) (0.2 mM) was used to induce enzyme expression for 8 h. The fermentation broth was then centrifuged at 4 °C, and the cells were collected, and then were resuspended in 50 mL of phosphate buffered saline (PBS) buffer. By ultrasonic disruption on ice the cells were lysed, and the cell debris was removed by centrifuging the lysate at 4 °C under 8000 *g* for 30 min. The supernatant was collected and transferred to a fresh tube, and then sodium chloride (3 M) was added to the tube. At 30 °C the solution was incubated for 10 min, and then centrifuged at 30 °C for 15 min. The purification process was repeated three times [18].

3.4. Solubility Measurement

HDP and HDP-ELP were separately dissolved in PBS. After incubations for 15, 30, 60, and 90 min, the samples were centrifuged for 30 min at 12,000 rpm. And then the amount of the enzymes remaining in the supernatants was measured by the bicinchoninic acid (BCA) method [24].

3.5. Western Blot

The expressed enzymes were separated on a 12% SDS-polyacrylamide gel. The separated enzymes were immersed for 15 min in a buffer (39 mM glycine, 48 mM Tris, 0.037% (*w*/*v*) SDS, 20% (*v*/*v*) methanol), and then were electro blotted to a nitrocellulose membrane (Sigma-Aldrich, Shanghai, China) using a Mini Trans-Blot Cell (Beijing Junyi Dongfang Electrophoresis Co., Beijing, China) according to a standard protocol [25]. Electrophoresis was conducted under a constant voltage of 200 V for 1.5 h, then the nitrocellulose membrane was washed with ultrapure water, and was then immersed in a solution of skimmed milk powder (5% (*w*/*w*)) for 1 h, the solution was prepared with blocking buffer (0.5% (*v*/*v*) Tween, 150 mM NaCl, 20 mM Tris-Hcl).Then the nitrocellulose membrane was incubated with anti His-tag mouse monoclonal antibody (dilution 1:1000) for 1 h. The nitrocellulose membrane was washed three times for 10 min each with blocking buffer. The secondary antibody was goat anti-rabbit IgG conjugated with horse radish peroxidase (HRP). Incubation of the nitrocellulose membrane with goat anti-rabbit IgG (dilution 1:1000) was carried out for 1 h. Then the nitrocellulose membrane was rinsed three times for 10 min each with blocking buffer. The reactive bands were visualized with enhanced chemo luminescence (ECL) Reagent.

3.6. CD Spectroscopy

CD spectra were measured on a JASCO J-810 CD instrument (JASCO Corporation, Shanghai, China). The bandwidth was 0.5 nm and the scan speed was 50 nm/min. Cell length was 1 cm. The enzyme solutions were prepared by dissolving the enzymes in PBS buffer. The HDP and HDP-ELP concentrations were 0.5 mg/mL. The measurements of CD spectra were carried out at 25 °C. The CD spectrum of the PBS buffer was used as control. By repeating five times scan, the averaged spectra were obtained.

3.7. Enzyme Assay and Analyses

The enzyme assay was carried out according to the description in the article [17]. Enzyme concentrations were determined by the BCA method [24]. The enzymes were dissolved in PBS buffer (50 mM, pH 8.0). For investigating the effects of the H_2O_2 concentration, temperature, and pH conditions on the enzymatic activity, the enzyme concentrations were 0.5 mg/mL, the initial L-tyrosine concentration was 1 mM. The change of L-tyrosine concentration with reaction time was monitored by HPLC (Shimadzu 15 LC-10A, Shimadzu, Shanghai, China), a C18 column (Diamonsil C18 250 × 4.6 mm, 5 μm, Dikma Technologies, Beijing, China) was used. The mobile phase was formic acid (0.08%, *v*/*v*)/acetonitrile (95:5, *v*/*v*) at a flow rate of 0.5 mL/min. All the solutions were filtered using a 220nm polycarbonate membrane prior to injection.

The enzyme kinetics for HDP-ELP was investigated at 30 °C. The HDP-ELP concentration was 0.15 mg/mL, the L-tyrosine concentrations were 0.125, 0.25, 0.37, 0.5, 0.75, 1.0, 1.5, 3.0 mM. The H_2O_2 concentration was 0.5 mM. The initial rate of reaction at each substrate concentration was determined by measuring the change of substrate concentration every 60 s for 5 min. At each substrate concentration, triplicate measurements were performed and averaged data were obtained. Kinetic parameters were determined by fitting the Michaelis–Menten equation to the initial rates of reactions.

4. Conclusions

The heme-dependent peroxidase has been genetically modified by fusing to an elastin-like polypeptide. The recombinant enzyme HDP-ELP exhibited a higher solubility in aqueous solutions

than HDP. CD spectra measurements showed that HDP-ELP has a higher stability than HDP. As a result, HDP-ELP exhibited a catalytic efficiency 2.4 times that of HDP. This work demonstrated that elastin-like polypeptides can be potentially fused to other peroxidases to improve their catalytic efficiency.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/6/11/166/s1, Table S1: ELP monomer, Table S2: The gene of the heme-dependent peroxidase, Figure S1: Plot of the initial rate of reaction (V_0) as a function of the substrate concentration for HDP-ELP, Figure S2: Effect of temperature and pH conditions on the enzymatic activity for HDP-ELP.

Acknowledgments: This work was supported by the National Science Foundation of China (21376021, 21576018).

Author Contributions: Wei Feng provided the idea and design for the study; Wei Liu and Rong Li performed the experiments; Dan Liu analyzed the data; Wei Liu drafted the manuscript; Wei Feng revised it.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Armstrong, R.D.; Hutchings, G.J.; Taylor, S.H. An overview of recent advances of the catalytic selective oxidation of ethane to oxygenates. *Catalysts* **2016**, *6*, 71. [CrossRef]
2. Ribitsch, D.; Yebra, A.O.; Zitzenbacher, S.; Wu, J.; Nowitsch, S.; Steinkellner, G.; Greimel, K.; Doliska, A.; Oberdorfer, G.; Gruber, C.C.; et al. Fusion of binding domains to thermobifida cellulosilytica cutinase to tune sorption characteristics and enhancing PET hydrolysis. *Biomacromolecules* **2013**, *14*, 1769–1776. [CrossRef] [PubMed]
3. Murugappan, G.; Zakir, M.J.A.; Jayakumar, G.C.; Khambhaty, Y.; Sreeram, K.J.; Rao, J.R. A novel approach to enzymatic unhairing and fiber opening of skin using enzymes immobilized on magnetite nanoparticles. *ACS Sustain. Chem. Eng.* **2016**, *4*, 828–834. [CrossRef]
4. Wang, Y.; Liu, C.; Zhang, Y.; Zhang, B.; Liu, J. Facile fabrication of flowerlike natural nanotube/layered double hydroxide composites as effective carrier for lysozyme immobilization. *ACS Sustain. Chem. Eng.* **2015**, *3*, 1183–1189. [CrossRef]
5. Rivero, C.W.; Palomo, J.M. Covalent immobilization of *Candida rugosa* lipase at alkaline pH and their application in the regioselective deprotection of per-*O*-acetylated thymidine. *Catalysts* **2016**, *6*, 115. [CrossRef]
6. Lee, J.H.; Nam, D.H.; Lee, S.H.; Park, J.H.; Park, S.J.; Lee, S.H.; Park, C.B.; Jeong, K.J. New platform for cytochrome P450 reaction combining in situ immobilization on biopolymer. *Bioconjugate Chem.* **2014**, *25*, 2101–2104. [CrossRef] [PubMed]
7. Lu, W.; Shen, Y.; Xie, A.; Zhang, W. Preparation and protein immobilization of magnetic dialdehyde starch nanoparticles. *J. Phys. Chem. B* **2013**, *117*, 3720–3725. [CrossRef] [PubMed]
8. Zhu, H.; Srivastava, R.; Brown, J.Q.; McShane, M.J. Combined physical and chemical immobilization of glucose oxidase in alginate microspheres improves stability of encapsulation and activity. *Bioconjugate Chem.* **2005**, *16*, 1451–1458. [CrossRef] [PubMed]
9. Mallardi, A.; Angarano, V.; Magliulo, M.; Torsi, L.; Palazzo, G. General approach to the immobilization of glycoenzyme chains inside calcium alginate beads for bioassay. *Anal. Chem.* **2015**, *87*, 11337–11344. [CrossRef] [PubMed]
10. Zhang, W.; Xu, F. Hierarchical composites promoting immobilization and stabilization of phytase via transesterification/silification of modulated alginate hydrogels. *ACS Sustain. Chem. Eng.* **2015**, *3*, 2694–2703. [CrossRef]
11. Wan, D.; Yuan, S.; Li, G.L.; Neoh, K.G.; Kang, E.T. Glucose biosensor from covalent immobilization of chitosan-coupled carbon nanotubes on polyaniline-modified gold electrode. *ACS Appl. Mater. Interfaces* **2010**, *2*, 3083–3091. [CrossRef] [PubMed]
12. Zhang, Y.; Ji, C. Electro-induced covalent cross-linking of chitosan and formation of chitosan hydrogel films: Its application as an enzyme immobilization matrix for use in a phenol sensor. *Anal. Chem.* **2010**, *82*, 5275–5281. [CrossRef] [PubMed]
13. Luo, X.; Zhang, L. Immobilization of penicillin G acylase in epoxy-activated magnetic cellulose microspheres for improvement of biocatalytic stability and activities. *Biomacromolecules* **2010**, *11*, 2896–2903. [CrossRef] [PubMed]

14. Mahmoud, K.A.; Lam, E.; Hrapovic, S.; Luong, J.H.T. Preparation of well-dispersed gold/magnetite nanoparticles embedded on cellulose nanocrystals for efficient immobilization of papain enzyme. *ACS Appl. Mater. Interfaces* **2013**, *5*, 4978–4985. [CrossRef] [PubMed]
15. Basu, S.; Omadjela, O.; Gaddes, D.; Tadigadapa, S.; Zimmer, J.; Catchmark, J.M. Cellulose microfibril formation by surface-tethered cellulose synthase enzymes. *ACS Nano* **2016**, *10*, 1896–1907. [CrossRef] [PubMed]
16. Nobre, T.M.; Silva, H.D.S.E.; Furriel, R.P.M.; Leone, F.A.; Miranda, P.B.; Zaniquelli, M.E.D. Molecular view of the interaction between ι-Carrageenan and a phospholipid film and its role in enzyme immobilization. *J. Phys. Chem. B* **2009**, *113*, 7491–7497. [CrossRef] [PubMed]
17. Connor, K.L.; Colabroy, K.L.; Gerratana, B. A hemeperoxidase with a functional role as an L-tyrosinehydroxylase in the biosynthesis of anthramycin. *Biochemistry* **2011**, *50*, 8926–8936. [CrossRef] [PubMed]
18. Meyer, D.E.; Chilkoti, A. Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: Examples from the elastin-like polypeptide system. *Biomacromolecules* **2002**, *3*, 357–367. [CrossRef] [PubMed]
19. Banki, M.R.; Feng, L.; Wood, D.W. Simple bioseparations using self-cleaving elastin-like polypeptide tags. *Nat. Methods* **2005**, *2*, 659–662. [CrossRef] [PubMed]
20. Hassouneh, W.; Fischer, K.; MacEwan, S.R.; Branscheid, R.; Fu, C.L.; Liu, R.; Schmidt, M.; Chilkoti, A. Unexpected multivalent display of proteins by temperature triggered self-assembly of elastin-like polypeptide block copolymers. *Biomacromolecules* **2012**, *13*, 1598–1605. [CrossRef] [PubMed]
21. Sun, J.; Du, K.; Song, X.; Gao, Q.; Ma, J.; Ji, P.; Feng, W. Specific immobilization of D-amino acid oxidase mimicking multi-enzyme catalysis. *Green Chem.* **2015**, *17*, 4465–4472. [CrossRef]
22. Conti, S.; Li, X.; Gianni, S.; Ghadami, S.A.; Buxbaum, J.; Cecchi, C.; Chiti, F.; Bemporad, F. A complex equilibrium among partially unfolded conformations in monomeric transthyretin. *Biochemistry* **2014**, *53*, 4381–4392. [CrossRef] [PubMed]
23. Schwaighofer, A.; Alcaráz, M.R.; Araman, C.; Goicoechea, H.; Lendl, B. External cavity-quantum cascade laser infrared spectroscopy for secondary structure analysis of proteins at low concentrations. *Sci. Rep.* **2016**. [CrossRef] [PubMed]
24. Smith, P.K.; Krohn, R.I.G.; Hermanson, G.; Klenk, D.C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76–85. [CrossRef]
25. Towbin, H.; Staehelin, T.; Gorgon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4350–4354. [CrossRef] [PubMed]

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