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Nitrospiropyran-modified α-chymotrypsin, a photostimulated biocatalyst in an organic solvent: effects of bioimprinting

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Abstract

α-Chymotrypsin (α-Chy) is modified with the nitrospiropyran active ester 1-(β-carboxyethyl-*N*-hydroxysuccinimide ester)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] (**1**) to yield a photoisomerizable enzyme. The modified enzyme is reversibly photoisomerizable between the nitrospiropyran state, α-Chy-SP, and the nitromerocyanine state, α-Chy-MR, that exists in an aqueous phase at neutral pH ≤ 8.0 in the protonated state, α-Chy-MRH$^+$. The two photoisomer states reveal in water similar biocatalytic activities for hydrolysis of *N*-acetyl-*L*-phenylalanine ethyl ester (**3**) to yield *N*-acetyl-*L*-phenylalanine (**2**). The photoisomerizable enzyme reveals photoswitchable activities in an organic phase consisting of cyclohexane, and esterification of **2** by ethanol is 3.4-fold faster in the presence of α-Chy-SP than by α-Chy-MR. Bioimprinting of the enzyme-substrate **2** into the biocatalyst via precipitation of the enzyme in the presence of the substrate from an aqueous solution yields a substantially more active biocatalyst in the organic phase. The bioimprinted photoisomerizable enzyme reveals photoswitchable biocatalytic activities in the organic phase, but the switching efficiency is lower than that observed for the non-imprinted biocatalyst. The bioimprinted α-Chy-SP is 2.2-fold more active than α-Chy-MR for hydrolysis of **3**. The lack of photoswitchable activities of the photoisomerizable enzyme in aqueous media compared to its photostimulated activities in the organic phase is attributed to the enhanced structural perturbation of the protein by the photoisomerizable units in the organic phase. The enhanced activity of the bioimprinted enzyme in the organic phase and its lower photoswitching efficiency compared to the non-imprinted photoisomerizable enzyme are attributed to the rigidification of the protein and its active site by the imprinting process. © 1997 Elsevier Science S.A.

Keywords: Photoswitchable enzyme; Bioimprinting; α-Chymotrypsin; Photoisomerizable materials; Nitrospiropyran

1. Introduction

Photostimulation of biomaterial functions to "ON" and "OFF" states is a rapidly progressing research area [1,2] directed to developing optobioelectronic systems [3], optical recording matrices [4] and biological computer units [5]. Other possible applications of photoswitchable biomaterials include the development of targeted, light-activated therapeutics [6,7] and reversible biosensor devices [8]. Different approaches to photostimulate biomaterials, i.e. enzymes or receptor proteins, have been discussed [2]. These include the chemical modification of proteins with photolabile [6,7] or photoisomerizable units [9–11], immobilization of enzymes in photoisomerizable supports, i.e. polymers [12], light-stimulated interaction of proteins with photoactive monolayers [13] and the use of photoisomerizable inhibitors [14,15].

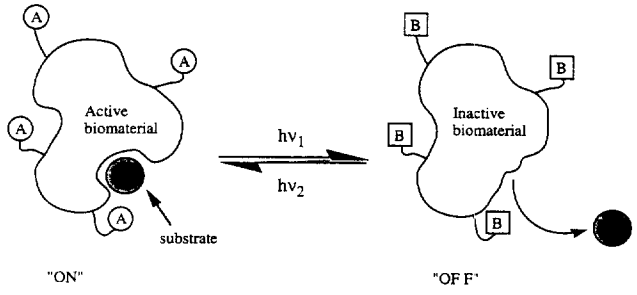

Fig. 1. Schematic representation of the photostimulated "ON–OFF" activities of a photoisomerizable enzyme.

The principle of photostimulation of protein functions by covalent attachment of photoisomerizable groups is schematically shown in Fig. 1. In one photoisomer state, A, the tertiary structure of the protein and its active-site is retained and the biomaterial is activated to perform its biochemical function. Photoisomerization to the second photoisomer state, B, results in the structural perturbation of the protein and its deactivation. This concept was successfully applied

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to reversibly photostimulate enzymes, i.e. papain, through covalent attachment of azobenzene units [9]. Using time-resolved light-scattering experiments, dynamic structural changes accompanying the photoisomerization of functionalized proteins were observed [16]. This concept was successfully applied to develop an optobioelectronic system by the chemical modification of glucose oxidase with photoisomerizable nitrospiropyran units [17]. The latter photostimulated redox-enzyme was applied for the amperometric transduction of recorded optical signals via the photoswitchable bioelectrocatalyzed oxidation of glucose. Nonetheless, this method yields only moderate switching efficiencies and the deactivated biomaterial exhibits a significant residual activity. It has been suggested that steric distortion of the protein by the photoisomerizable units is insufficient to cause entire blocking of the biomaterial [9,17].

The application of enzymes in organic media is a common practice in biocatalysis [18–20]. It has been shown that the tertiary structure of enzymes is retained in the organic phase by the co-association of water molecules that bridge and stabilize the protein backbone. It was further demonstrated that bioimprinting the biocatalyst-substrate into the enzyme, upon precipitation of the enzyme from an aqueous solution, rigidified the active-site microenvironment and the biocatalyst exhibits improved activities in organic media [21]. It has been suggested [22] that photostimulated enzymes, formed by covalent attachment of photoisomerizable units to the protein, could reveal enhanced switching efficiencies by their application in organic solvents. As the content of water associated with the protein is limited in organic solvents, the structural perturbation of the protein upon photoisomerization should be pronounced and hence improved photoswitchable activities are anticipated. Indeed, it was demonstrated that the photoisomerizable thiophenefulgide α-chymotrypsin is not photostimulated in an aqueous phase, but reveals photoswitchable activities in an organic solvent [22].

Here we wish to report on the generality of this approach by evaluating the photostimulated properties of nitrospiropyran-modified α-chymotrypsin. We address the improved switching efficiencies of the photoisomerizable enzyme in an organic medium (cyclohexane) as compared to an aqueous phase and discuss the effects of substrate-bioimprinting on the switching efficiency of the biocatalyst.

2. Experimental

Absorption spectra were recorded on a Uvikon-860 (Kontron) spectrophotometer. High-performance liquid chromatography (HPLC) analyses were performed on a Merck Hitachi liquid chromatograph equipped with a UV detector. α-Chymotrypsin (E.C. 3.4.21.1) and all other chemicals, unless otherwise stated, were from commercial sources (Sigma, Aldrich) and were used without further purification. Ultrapure water from a Nanopure (Barnstead) source was used.

The nitrospiropyran active ester (**1**) 1-(β-carboxyethyl-*N*-hydroxysuccinimide ester)-3,3-dimethyl-6′-nitrospiro-[indoline-2,2′-2*H*-benzopyran] was prepared by coupling the parent carboxylic acid 1-(β-carboxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzopyran] with *N*-hydroxysuccinimide according to the general reported method [23]. The parent acid was prepared according to the literature [24].

Chemical modification of α-chymotrypsin by nitrospiropyran units was carried out according to the following procedure: 100 mg of α-chymotrypsin were dissolved in 12 ml of an aqueous solution that contained 500 mg $NaHCO_3$. A solution of 40 mg of **1** in tetrahydrofuran (400 μl) was added to the aqueous enzyme solution and the resulting mixture was allowed to react at 4 °C for 48 h. The mixture was dialyzed and the resulting solution was lyophilized to dryness. The average loading of the protein by nitrospiropyran units was determined by photoisomerization of a known weight of the modified protein to the nitromerocyanine state, α-Chy-MRH^+. The absorbance spectrum of the resulting solution was recorded and the bulk concentration of MRH^+ units was determined by following the absorbance at $\lambda = 510$ nm ($\varepsilon = 2300\ M^{-1}\ cm^{-1}$).

Bioimprinting of α-Chy-SP was performed with the substrate *N*-acetyl-*L*-phenylalanine (**2**). The biocatalyst α-Chy-SP (30 mg) was dissolved in 1 ml of sodium phosphate buffer (1×10^{-2} M, pH = 7.8) that contained **2** (2×10^{-2} M). The enzyme solution was cooled to 0 °C and equilibrated for 2 h. *n*-Propanol, precooled to −20 °C (4 ml), was then added to the solution. The resulting precipitate was washed three times with *n*-propanol followed by centrifugation and removal of the solvent, and dried under vacuum.

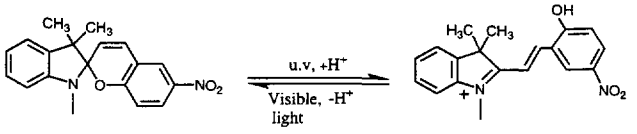
Assaying the activities of α-chymotrypsin and of α-Chy-SP and α-Chy-MRH^+ in cyclohexane was performed by the following procedure: 8 mg of the respective enzyme in imprinted or non-imprinted form were suspended in 2 ml of cyclohexane that contained 0.5 ml of an ethanol solution of **2** (final concentration of the substrate 2×10^{-2} M). Samples (80 μl) were taken from the reaction mixture at time intervals and introduced into vials that contained a 9-mm RC 58 0.2 μm filter (Schleicher and Schuell). The vials were centrifuged to filter off the enzyme, and the reaction samples were analyzed by HPLC (RP-18 column, eluent solution methanol–water 65:35%, 0.8 ml min^{-1}, UV detection, $\lambda = 254$ nm).

Photochemical experiments were performed by illumination of the reaction mixture in a thermostated bath (20 °C) using appropriate light sources. For the generation of the α-Chy-SP state, a 150 W xenon lamp equipped with a filter ($\lambda > 475$ nm) was used. For the generation of the α-Chy-MRH^+ state, an Hg pencil-lamp source (Oriel, 6042 long-wave filter) 320 nm $< \lambda <$ 380 nm was used. Illumination at the various wavelengths was applied continuously during the experiments to eliminate thermal isomerization of α-Chy-SP to α-Chy-MRH^+ or uncontrolled roomlight isomerization of α-Chy-MRH^+ to α-Chy-SP.

Hydrolysis of *N*-acetyl-l -phenylalanine ethyl ester (**3**) was assayed by the two isomer states α-Chy-SP and α-Chy-MRH$^+$, respectively. The modified α-chymotrypsin, α-Chy-SP or α-Chy-MRH$^+$ (1.2 mg), was introduced into 2.4 ml of water. The substrate **3** was dissolved in 100 μl acetonitrile and injected into the enzyme aqueous solution (2×10^{-2} M), and the rate of formation of the product (**2**) was followed by HPLC. The enzyme isomer states, α-Chy-SP or α-Chy-MRH$^+$, were generated by illumination with the respective light source of the enzyme aqueous solution for 15 min at 22 °C.

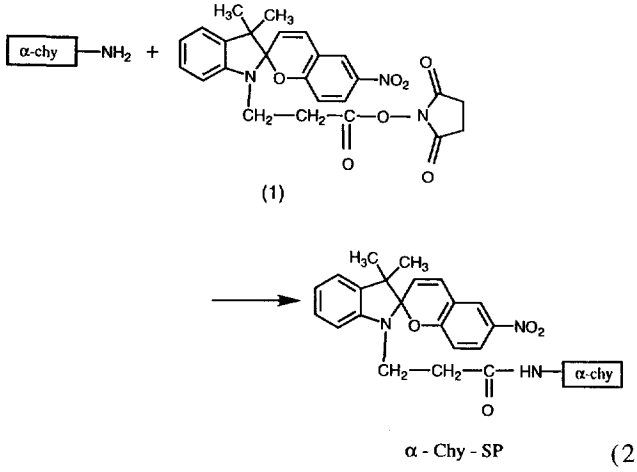
3. Results and discussion

Nitrospiropyran (SP), derivatives and related compounds exhibit well-characterized reversible photoisomerizable properties [25]. Irradiation of nitrospiropyran compounds with UV light yields the zwitterionic nitromerocyanine isomer, the MR state. In aqueous media, the nitromerocyanine state exists at pH ≤ 8.0 in the protonated state, the MRH$^+$ state, (Eq. (1)). Photoirradiation of the nitromerocyanine isomer state (or the protonated MRH$^+$ state) with visible light regenerates the SP state. The latter process is also thermally stimulated.



SP MRH$^+$ (1)

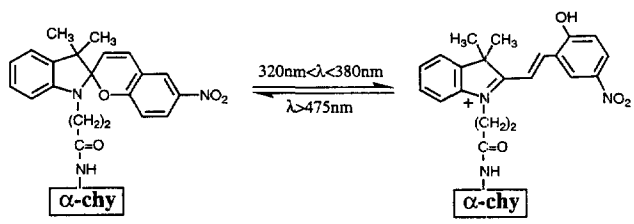
α-Chymotrypsin (α-Chy) was modified with nitrospiropyran units by covalent attachment of 1-(β-carboxyethyl-*N*-hydroxysuccinimide ester)-3,3′-dimethyl-nitrospiro-[indoline-2,2′-2H-benzopyran] (**1**) to lysine residues of the protein (Eq. (2)).



(1)

α - Chy - SP (2)

The resulting nitrospiropyran-modified α-chymotrypsin, α-Chy-SP, exhibits reversible photoisomerizable properties (Eq. (3)).



α - Chy - SP α - Chy - MRH$^+$ (3)

Irradiation of α-Chy-SP, exhibiting the spectrum shown in Fig. 2(a), with UV light, 320 nm < λ < 380 nm, yields the protonated nitromerocyanine α-chymotrypsin, α-Chy-MRH$^+$ state, Fig. 2(b). Further illumination of the α-Chy-MRH$^+$ with visible light, λ > 475 nm, regenerates α-Chy-SP. The average loading of the protein by nitrospiropyran units was determined by the transformation of a known amount of the modified protein to the α-Chy-MRH$^+$ state. By following the absorbance of the MRH$^+$ units at λ = 510 nm ($\varepsilon = 2300$ M^{-1} cm^{-1}) the bulk concentration of the photoisomerizable substrate was evaluated, and related to the concentration of the protein. The average loading of α-chymotrypsin by nitrospiropyran units, was found to correspond to 6.

Bioimprinting of α-Chy-SP was performed by the treatment of the protein with its substrate *N*-acetyl-l -phenylalanine (**2**) in an aqueous medium, followed by precipitation and removal of the imprinted substrate. The activity of the bioimprinted α-Chy-SP was compared to that of bioimprinted native α-chymotrypsin by following the rate of esterification of **2** by ethanol in cyclohexane (Eq. (4)). The modified enzyme α-Chy-SP retains 80% of the activity of the native enzyme.

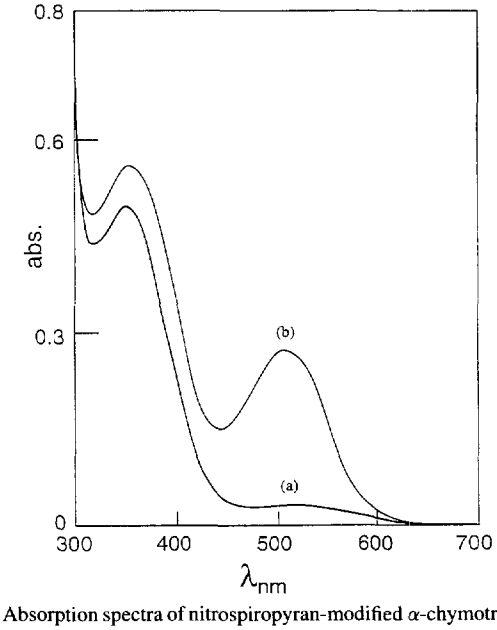
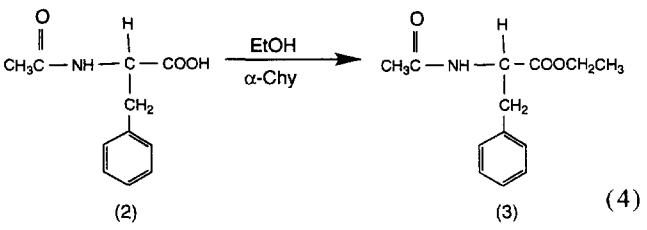


Fig. 2. Absorption spectra of nitrospiropyran-modified α-chymotrypsin (0.5 mg ml^{-1}) in phosphate buffer solution, pH = 7.8: (a) α-Chy-SP; (b) α-Chy-MRH$^+$.



(4)

The activities of the two photoisomer states of modified α-chymotrypsin, α-Chy-SP and α-Chy-MR, were characterized by following the rates of esterification of **2** by ethanol in cyclohexane to form **3** upon the application of a non-bioimprinted biocatalyst and a bioimprinted biocatalyst. Fig. 3 shows the rate of esterification of **2** by ethanol in the presence of the non-bioimprinted α-Chy-MR and α-Chy-SP, curves (a) and (b), respectively. The rate of esterification of **2** by ethanol with the substrate-bioimprinted enzyme in the forms α-Chy-MR and α-Chy-SP is shown in Fig. 3, curves (c) and (d), respectively. It is evident that the substrate-bioimprinted enzyme α-Chy-SP is about 4-fold more active than the non-bioimprinted α-Chy-SP. Similarly, the substrate-bioimprinted α-Chy-MR reveals an approximately 5.5-fold enhanced activity compared to the non-bioimprinted biocatalyst. These results are consistent with the fact that bioimprinting of the enzyme with its reaction substrate, through precipitation from the aqueous phase, rigidifies the active-site microenvironment that is preserved in the organic medium. This rigidification of the active-site structure leads to the enhanced activities of the bioimprinted enzymes in cyclohexane. Furthermore, the results demonstrate that in either substrate-bioimprinted or non-bioimprinted enzymes the α-Chy-SP state reveals higher activities than the α-Chy-MR state. This suggests that the activity of the biocatalyst could be photostimulated by photoisomerization of the chemical units linked to the protein.

Fig. 4(a) shows the photostimulated esterification of **2** in cyclohexane by the substrate-bioimprinted photoisomerizable α-chymotrypsin. The experiment is initiated in the presence of α-Chy-MR, where a slow esterification of **2** is

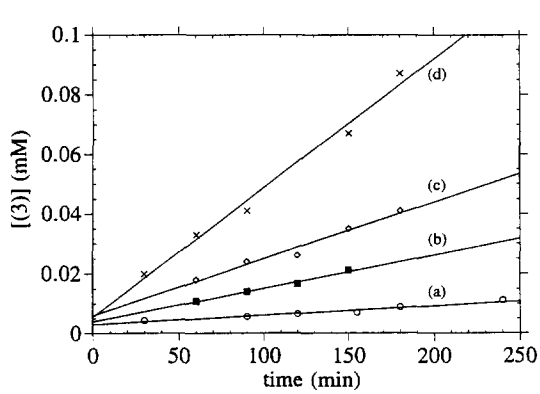


Fig. 3. Rates of esterification of *N*-acetyl-L-phenylalanine (**2**) (2×10^{-2} M) by ethanol (0.5 ml) in cyclohexane (2 ml) using modified α-chymotrypsin (8 mg): non-bioimprinted α-Chy-MR (a) and α-Chy-SP (b); bioimprinted α-Chy-MR (c) and α-Chy-SP (d).

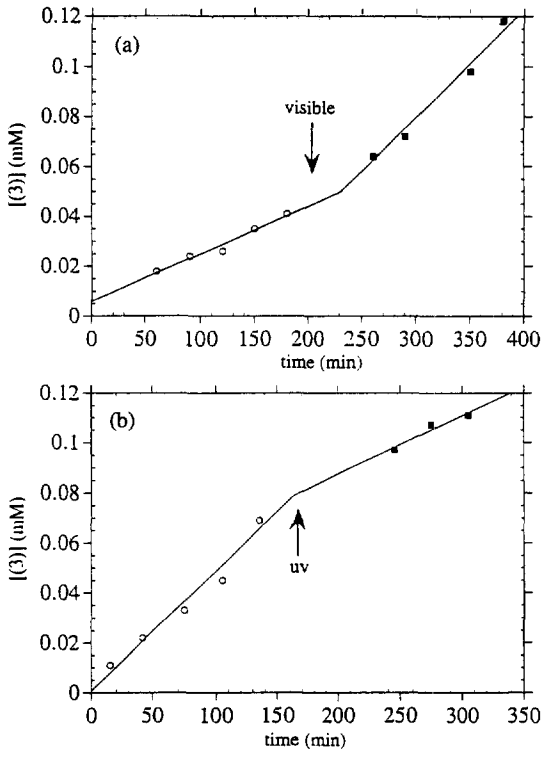


Fig. 4. Photostimulated rates of esterification of *N*-acetyl-L-phenylalanine (**2**) (2×10^{-2} M) by ethanol (0.5 ml) in cyclohexane (2 ml) using the bioimprinted photoisomerizable α-chymotrypsin (8 mg): (A) esterification is initiated by the enzyme in the α-Chy-MR state. Arrow indicates photoisomerization of α-Chy-MR to α-Chy-SP state (switch "ON"); (B) esterification is initiated with the α-Chy-SP state. Arrow indicates photoisomerization of α-Chy-SP to α-Chy-MR state (switch "OFF").

observed. At the time marked with an arrow, the biocatalyst is photoisomerized to the α-Chy-SP state, using visible light, $\lambda > 475$ nm. This results in an enhancement in the esterification rate of **2**. The photostimulated activities of the biocatalyst can be reversed in their direction as shown in Fig. 4(b). Here the experiment is initiated with α-Chy-SP and a fast esterification of **2** is observed. At the time marked with an arrow, the biocatalyst is photoisomerized to the α-Chy-MR state, using UV light, 320 nm $< \lambda <$ 380 nm. This results in a retardation in the rate of esterification of **2**. The photostimulated switching efficiency of the photoisomerizable, substrate-bioimprinted biocatalyst, defined as $\nu_{\alpha\text{-Chy-SP}}/\nu_{\alpha\text{-Chy-MR}}$ corresponds to 2.2.

The photostimulated activation of the non-bioimprinted photoisomerizable biocatalyst was also examined, Fig. 5. Here the experiment is initiated with the α-Chy-MR biocatalyst and a slow esterification of **2** is observed. Upon photoisomerization to the α-Chy-SP state, $\lambda > 475$ nm, (time marked with an arrow), the process is substantially enhanced. The photostimulated switching efficiency of the non-bioimprinted biocatalyst corresponds to 3.4. We realize that for the substrate-bioimprinted or non-imprinted enzymes the α-Chy-SP state is always more active than the α-Chy-MR state. The switching efficiency ($\nu_{\alpha\text{-Chy-SP}}/\nu_{\alpha\text{-Chy-MR}}$) is higher for the non-bioimprinted enzyme as compared to the substrate-

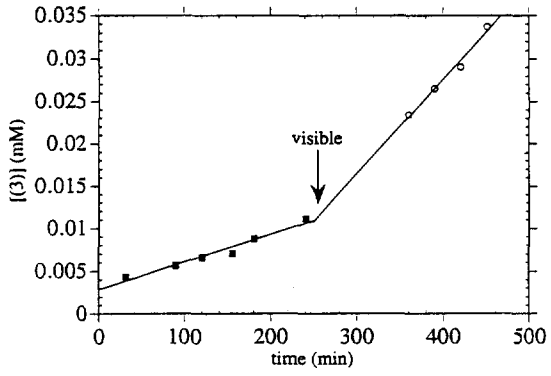


Fig. 5. Photostimulated rates of esterification of *N*-acetyl-l -phenylalanine (**2**) (2×10^{-2} M) by ethanol (0.5 ml) in cyclohexane (2 ml) using the non-bioimprinted photoisomerizable α-chymotrypsin (8 mg). Esterification is initiated by the enzyme in the α-Chy-MR state. Arrow indicates photoisomerization of α-Chy-MR to α-Chy-SP state.

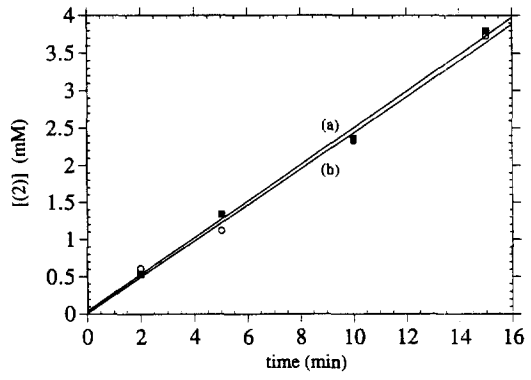
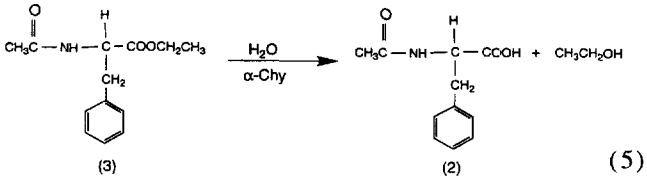


Fig. 6. Hydrolysis rate of *N*-acetyl-l -phenylalanine ethyl ester (**3**) by the photoisomerizable enzyme in an aqueous buffer solution, pH = 7.0: (a) by α-Chy-SP; (b) by α-Chy-MRH$^+$.

bioimprinted biocatalyst. Molecular mechanics calculations of nitrospiropyran and nitromerocyanine reveal that the photoisomerization leads to significant structural changes. While the nitrospiropyran state is sterically compact, the nitromerocyanine is sterically elongated. Thus, steric perturbation of the tertiary structure of the enzyme, and specifically of the active-site microenvironment by the MR units, is suggested to lead to the lower activity of the enzyme, α-Chy-MR. In the α-Chy-SP state, the photoisomerizable components have only a minor effect on the protein structure and this yields the high activity of this photoisomer state. In addition to the steric differences between the nitrospiropyran and nitromerocyanine states, the latter photoisomer is in a zwitterionic structure. Electrostatic attractive or repulsive interactions between the photoisomerizable components and the protein could further add a driving force for the structural distortion of the biocatalyst in the α-Chy-MR state. The higher switching efficiency of the non-bioimprinted enzyme as compared to the bioimprinted biocatalyst can be attributed to the general effect of substrate-bioimprinting on the protein structure in organic solvents. The protein is stabilized in the organic medium by the co-association of water molecules to the protein. This water content retains and preserves the tertiary structure of the protein. The substrate-bioimprinting process rigidifies the active-site structure, and yields a biocatalyst of improved performance in the organic phase. Photoisomerization of the α-Chy-SP biocatalyst to the α-Chy-MR state results in only moderate perturbation of the active-site microenvironment, due to its initial rigidification by the bioimprinting process. For the non-imprinted enzyme, the active-site is non-rigidified in the organic solvent and hence the structural distortion of the protein by photoisomerization of α-Chy-SP to α-Chy-MR is enhanced. This leads to the improved switching efficiencies observed for the non-imprinted enzyme.

A further aspect to consider involves the photostimulated activities of the photoisomerizable enzyme in an aqueous medium. Fig. 6 shows the rates of hydrolysis of *N*-acetyl-l -phenylalanine ethyl ester (**3**) in water to form **2** (Eq. (5)), in the presence of α-Chy-SP and α-Chy-MRH$^+$. The two enzyme states reveal similar biocatalytic activities in the aqueous solution.



(5)

The lack of photoswitchable activities of the photoisomerizable enzyme in aqueous media should be compared to the high switching efficiencies of the photoisomerizable biocatalysts in either bioimprinted or non-bioimprinted configurations. The marked differences can be attributed to the extent of stabilization of the protein in the aqueous phase vs. the organic phase, and the influence of the photoisomerizable components on the protein structure. In the aqueous medium, the enzyme is stabilized by water molecules bridging the protein backbone. The photoisomerization of α-Chy-SP to α-Chy-MRH$^+$ induces only a slight structural perturbation of the protein due to its stabilization by bridging water molecules. In the organic phase, only a limited amount of water molecules is associated with the protein. This results in the pronounced structural distortion of the protein upon photoisomerization of α-Chy-SP to α-Chy-MR.

4. Conclusions

We have prepared a nitrospiropyran-modified α-chymotrypsin as a photoisomerizable biocatalyst and have characterized its photostimulated activities in cyclohexane. The effects of substrate-bioimprinting on the enzyme activities and switching efficiencies were elucidated. We observed that the photoisomerizable enzyme exhibits photostimulated activities in cyclohexane. The esterification rate of **2** by ethanol is fast in the presence of α-Chy-SP and retarded with α-Chy-MR. The decreased activities of α-Chy-MR are attributed to structural distortion of the protein and the active-site microenvironment as a result of the isomerization of the SP to MR units. The substrate-bioimprinted enzyme reveals

higher activities for esterification of **2** in cyclohexane as compared to the non-bioimprinted biocatalyst. This is attributed to the rigidification and stabilization of the active-site by the imprinting process. However, the substrate-bioimprinted biocatalyst reveals a lower switching efficiency (2.2) as compared to the non-bioimprinted enzyme (3.4). This is attributed to the stabilization of the active-site via the bioimprinting process. The structural perturbation of the bioimprinted enzyme by photoisomerization of α-Chy-SP to α-Chy-MR is limited as compared to that occurring in the non-imprinted biocatalyst. This yields the lower switching efficiency of the former photoisomerizable enzyme. The photoisomerizable enzyme lacks photoswitchable activities in the aqueous phase. This was attributed to stabilization of the protein structure in the aqueous medium that prevents significant structural perturbations by the photoisomerizable units.

Acknowledgements

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