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RESEARCH ARTICLE

Reversible immobilization of cephalosporin C acylase on epoxy supports coated with polyethyleneimine

YANMEI WEI[1,2], HUI LUO[1], YANHONG CHANG[2], HUIMIN YU[3] & ZHONGYAO SHEN[3]

[1]*School of Chemistry and Biological Engineering, University of Science and Technology Beijing, Beijing, PR China,* [2]*School of Civil and Environmental Engineering, University of Science and Technology Beijing, Beijing, PR China, and* [3]*Department of Chemical Engineering, Tsinghua University, Beijing, PR China*

Abstract

In this study, a recombinant cephalosporin C acylase (CCA) was covalently or physically immobilized on an epoxy-activated support LX1000-EPC4 (EP) or its derivatives, EP-polyethyleneimine (EP-PEI) and EP-ethylenediamine (EP-EDA) with cationic groups on the surface. Zeta potential was used as a tool for activated carrier analysis and immobilization analysis. The EP-PEI (the cationic polymer PEI grafted support) showed higher zeta potential than EP-EDA (the small diamine EDA modified support) and EP support. Among these three supports, immobilization of CCA on EP-PEI had the highest specific activity according to the range of enzyme loadings. Michaelis constant K_m values of EP-PEI-CCA and EP-EDA-CCA were 22 mM and 30 mM, respectively, which were lower than that of the free enzyme (43 mM), suggesting that the support's zeta potential is related to the affinity of the enzyme for the substrate. The enzyme immobilized on EP-PEI showed a much higher thermal stability (stabilization factor of 32-fold compared with the free enzyme) than that on the EP-EDA (stabilization factor of 5.5-fold) and EP supports (stabilization factor of 1.7-fold). The adsorption of CCA on EP-PEI support was very strong and reversible. The CCA could be thoroughly desorbed using a high concentration of NaCl (e.g., 2 M) at low pH value (pH 3.0). The regenerated EP-PEI support could then be reused for enzyme immobilization.

Keyword: *Cephalosporin C acylase*, *polyethyleneimine*, *reversible immobilization*, *zeta potential*

Introduction

Cephalosporin C acylase (CCA) is a novel enzyme that directly hydrolyzes cephalosporin C to 7-aminocephalosporanic acid (7-ACA), a key intermediate in the production of semi-synthetic cephalosporin antibiotics (Pollegioni et al. 2013). Although 7-ACA is currently produced by a two-step enzymatic process with D-amino acid oxidase and glutaryl acylase, there are some inherent problems, for example, the deleterious effect of hydrogen peroxide, the byproduct of D-amino acid oxidase catalysis, on enzyme stability, and product yield (López-Gallego et al. 2008; Volpato et al. 2010). CCA shows great potential for industrial 7-ACA production because of the advantages of simplicity, high efficiency, and low cost (Volpato et al. 2010; Pollegioni et al. 2013).

When properly designed, immobilized enzymes often have greater thermal and operational stability than the soluble form of the enzyme (Garcia-Galan et al. 2011; Barbosa et al. 2013). In most industrial biocatalytic processes, the advantages of immobilized enzymes over soluble enzymes arise from their enhanced stability and ease of separation from the reaction media, leading to significant savings in enzyme consumption (Tran and Balkus 2011; Barbosa et al. 2013; Rodrigues et al. 2013).

Among the traditional enzyme immobilization protocols, physical adsorption (Daunert et al. 2007; Filho et al. 2008; López-Gallego et al. 2012) and covalent bonding (Mateo et al. 2000; Garcia-Galan et al. 2011) are often used. Enzyme immobilization by means of physical adsorption (usually via ionic or hydrophobic interactions) is a mild process with high retention of activity (Mateo et al. 2006; Marquez

Correspondence: Hui Luo, School of Chemistry and Biological Engineering, University of Science and Technology Beijing, Beijing 100083, PR China. Tel: +86 1082377396. E-mail: luohui@ustb.edu.cn

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et al. 2008; Cantone et al. 2013; Jesionowski et al. 2014). However, enzyme desorption from the supports (e.g., ionic exchangers) is a crucial issue to be considered at high ionic strength and pH extremes (Filho et al. 2008; López-Gallego et al. 2012).

As an alternative to physical adsorption, immobilization via covalent bonding irreversibly attaches the enzyme with no protein leakage during enzyme utilization; biocatalysts often have good stability and reusability due to the strong covalent bonds between the enzyme and support (Mateo et al. 2007a; Barbosa et al. 2014). For example, immobilizing enzymes via a two-step mechanism using epoxy-activated supports, can increase conformational stability by favoring additional multipoint covalent attachment of the enzyme (Mateo et al. 2000; Mateo et al. 2007a). Nevertheless, enzymes immobilized via covalent bonding may undergo conformational changes, which affect the active site due to the severe reaction conditions during immobilization. This often leads to a relatively low activity recovery rate (Mateo et al. 2000; Zhu et al. 2011).

Several protocols for reversible immobilization of proteins have been described (Batista-Viera et al. 1991; Fuentes et al. 2004; Daunert et al. 2007; López-Gallego et al. 2012). The adsorption of enzymes on ion exchangers is the most common and easy protocol for reversible immobilization with facile regeneration of the supports (Pessela et al. 2006). However, immobilization of enzymes on traditional ion exchangers is often weak and most proteins are released from the carriers if the pH value changes or the ionic strength increases (Filho et al. 2008; Liu et al. 2012).

Unlike traditional ion exchangers, solid matrix grafted with cationic polymers (e.g., polyethyleneimine, PEI) has been proposed as a possible support for strong but reversible immobilization of enzymes (Pessela et al. 2006). PEI is a branched polymer with many amino groups, and supports grafted with PEI offer a three dimensional surface for interaction with enzymes via multipoint adsorption (Pessela et al. 2004; Torres et al. 2006). Thus, grafted PEI with a high charge density enables carriers to adsorb enzymes more strongly than the usual ion exchangers and prevents enzyme desorption (Pessela et al. 2003).

The adsorption of an enzyme to a support depends not only on the nature of the chemical interactions formed, but also on the surface charge of the support. Zeta potential measures the charge on the surface of solid powder particles in suspension, and this value of surface charge is useful for understanding and predicting interactions between particles in suspension (Yukselen and Kaya 2003; Yukselen-Aksoy and Kaya 2011). Therefore, the zeta potential, which quantifies the electrostatic interactions between enzyme and support, could be a diagnostic tool for binding efficiency in enzyme immobilization studies (Schultz et al. 2008). Surprisingly, there are few reports considering the zeta potential of the supports let alone the zeta potential of the immobilized enzyme in the process of immobilization (Ding et al. 2005; Gomez et al. 2012).

CCA is a useful enzyme for the industrial production of 7-ACA. The stability and cost of the immobilized enzyme are therefore vital for its application. In previous reports, CCA was covalently immobilized on epoxy-activated supports and the CPC catalyzing efficiency of the catalyst was verified (Boniello et al. 2010; Zhu et al. 2011). Nevertheless, with CCA being a heterodimeric enzyme, which is probably deactivated during catalysis, further efforts are needed to improve stability and economic aspects of biocatalysts to meet industrial requirements (Cantone et al. 2013; Luo et al. 2014; He et al. 2015). Based on the analysis given above, the reversible immobilization of enzymes on PEI coating supports might increase biocatalyst stability and simultaneously decrease the cost of supports due to re-use of the support matrix (Torres et al. 2002).

In this work, ion exchange supports were derived from the commercial epoxy-activated support LX-1000EPC4 and used for CCA reversible immobilization. To study the immobilization of CCA, zeta potential was used to assess protein binding on different supports and characteristics of the immobilized enzymes. Reversible immobilization of CCA on the supports and their reusability was also investigated.

Materials and methods

Materials

CPC and 7-ACA were kindly supplied by North China Pharmaceutical Co. Ltd. (Shijiazhuang, PR China). The epoxy support LX-1000EPC4 (EP) (with an epoxy group content around 60 μmol/g, the particle size of 150–300 μm, the average pore size around 15 nm) was kindly supplied by Sunresin New Materials Co. Ltd. (Xi'an, PR China). Polyethyleneimine (PEI, M_w 20,000) was purchased from Aladdin Industrial Inc. Other reagents were of analytical grade purchased from the local market.

Preparation of CCA

CCA was prepared from cultivation of recombinant *E. coli* BL21(DE3)/pET28-CPCAcy (Zhu et al. 2011). The harvested cells were suspended in sodium phosphate buffer (0.02 M, pH 8.0) and cell extracts were prepared by sonication and centrifuged.

Then the 6 histidine-tagged CCA was purified to get purity higher than 90% by means of immobilized metal ion affinity chromatography (IMAC) as described previously (Zhu et al. 2011). The specific activity of the loaded CCA solution was 9.2 U/mg.

Assay of CCA activity

The enzyme activity of CCA against CPC was measured according to a previously described method (Zhu et al. 2011). One unit of CCA activity is defined as the amount of enzyme capable of producing 1 μmol of 7-ACA per minute at 37 °C and pH 8.5.

The specific activity of the immobilized enzyme was determined under the previously described conditions and was defined as 1 μmol of 7-ACA produced per minute per gram of wet immobilisate. All data are the average value of at least three experiments. The experimental error was less than 5%.

Preparation of supports by modifying EP with PEI and ethylenediamine (EDA)

Modification of the epoxy support EP with PEI was performed as previously reported, and a derivative named EP-PEI was obtained (Rocchietti et al. 2004). The epoxy support was also modified as previously described with a small diamine EDA to obtain the derivative EP-EDA, which is very similar to the traditional cationic exchanger (Mateo et al. 2007b).

Immobilization of CCA on EP support

The epoxy support LX-1000EPC4 was suspended in 1.25 M sodium phosphate buffer at pH 8.0 and 25 °C. A suitable amount of CCA solution was added to give a final concentration of sodium phosphate around 0.85 M and the immobilization mixture was kept under mild stirring for 24 h. Then the derivative was filtered and washed with sodium phosphate buffer (0.1 M, pH 8.5) and stored at 4 °C for further use. Finally, the remaining epoxy groups were blocked with 3 M glycine in pH 8.0 buffer at 25 °C for 24 h (Mateo et al. 2002). To produce multipoint attachment of CCA on the epoxy support, the EP-CCA preparation was treated under conditions (in an pH 10 phosphate buffer containing 0.1 M 7-ACA and 20% glycerol, at 25 °C for 24 h), similar to that applied in the reference for the immobilization of penicillin G acylase (Mateo et al. 2000).

Immobilization of CCA on EP-PEI and EP-EDA supports

The EP-PEI or EP-EDA supports were suspended in 5 mM sodium phosphate buffer at pH 8.0 and 25 °C with addition of a suitable amount of CCA solution, and the mixture kept under mild stirring for 30 min (Rocchietti et al. 2004; Mateo et al. 2007b). Then the preparation was washed with sodium phosphate buffer (0.1 M, pH 8.5) and stored at 4 °C for further use.

Detection of zeta potential

To meet the particle size limitation of the device for zeta potential measurement, different supports were prepared by grinding them to the fine particles and passing them through a 325-mesh sieve, and then the same immobilization procedure was performed according to the method described in section 2.5 and 2.6. The zeta potential of the different CCA preparations and supports was measured with a ZetaPlus instrument (Brookhaven Instruments Corporation, Holtsville, NY).

Thermal stability of different CCA preparations

Enzyme preparations were incubated at 45 °C in 0.02 M at pH 8.0 phosphate buffer. Samples were periodically withdrawn, and their activities tested as described. The CCA residual activity was expressed as a percentage of the initial activity at the given incubation time.

Desorption of enzyme from different ionic supports

The immobilized CCA (the CCA preparations with enzyme loading of 100 U/g support) was incubated in 5 mM sodium phosphate buffer (pH 8.0) with increasing concentration of NaCl at 25 °C, and aliquots of the suspension were periodically collected for activity analysis of the desorbed enzyme.

Kinetic parameters of the CCA preparations

The initial reaction rates of CPC hydrolysis with free and immobilized CCA (the CCA preparations with enzyme loading of 100 U/g support) were determined at different CPC concentrations (4–20 mg/ml). The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) for the free and immobilized enzyme were calculated according to Lineweaver-Burk plots.

Reusability of EP-PEI support

To investigate the reusability of the EP-PEI support, the CCA was desorbed from the EP-PEI support with 2 M NaCl (pH 3.0) for 30 min at 25 °C (Wu et al. 2012), and then the regenerated support

was reused for a new immobilization procedure according to section ''Immobilization of CCA on EP-PEI and EP-EDA supports''. In the repeated adsorption test, the CCA-loading activity in each cycle was 100 units per gram support.

Results and discussion

Determination of zeta potential

To investigate the impact of zeta potential on the interaction of the support and the enzyme during immobilization, CCA was covalently or physically immobilized on different supports, such as EP, EP-PEI, and EP-EDA. EP-EDA and EP-PEI were derived from EP by modifying it with the small molecule EDA and cationic polymer PEI, respectively. It should be pointed out that the particles were ground and sieved to less than 50 μm to meet the requirement of particle size limitation of the device for Zeta potential measurement. The ground particle should have the same properties as the initial support for enzyme immobilization, except particle size. Thus, to obtain the zeta potential data representative of the unbroken support, enzyme immobilization on the ground support was performed under the same conditions of protein loading and immobilization time. Thanks to the increased surface area of the particle and reduced mass transfer limitation, the ground support gave a higher immobilization yield and specific activity than the unbroken one (data not shown). Though the particle size would affect immobilization parameters, the different zeta potentials of ground supports prepared under the same conditions can be used for comparison of immobilization differences for different supports.

The zeta potential of different supports and CCA preparations with small particle sizes is shown in Table 1. The zeta potential of CCA was found to be −11.28 mV in pH 8.5 phosphate buffer, indicating that CCA has a net negative charge when used in catalysis or immobilization. The substrate CPC has a similar negative charge in pH 8.5 solution.

The hydrophobic polyacrylate-based epoxy support was electrically neutral in phosphate buffer. The EP-PEI and EP-EDA, derivatives of the EP support, however, carried a positive charge because of the cationic groups from the modifying reagents. Since zeta potential is a function of the surface charge of a particle and the nature and composition of the surrounding medium in which the particle is suspended, the high density of amino groups on the surface of EP-PEI resulted in the highest zeta potential of the three supports. Therefore, the EP-PEI support should easily adsorb an enzyme carrying a negative charge via electrostatic interaction in solution.

Table 1. Zeta potential of different CCA and different supports.

Sample	Medium	Zeta potential(mV)
Free enzyme[a]	Buffer (0.1 M, pH 8.5)	−10.98
CPC-Na[b]	Buffer (0.1 M, pH 8.5)	−10.06
EP	Deionized water	−0.57
EP	Buffer (0.1 M, pH 8.5)	0
EP-CCA[c,f]	Buffer (0.1 M, pH 8.5)	−11.59
EP-EDA	Deionized water	0.25
EP-EDA	Buffer (0.1 M, pH 8.5)	0.39
EP-EDA-CCA[d,f]	Buffer (0.1 M, pH 8.5)	−9.21
EP-PEI	Deionized water	0.57
EP-PEI	Buffer (0.1 M, pH 8.5)	0.65
EP-PEI-CCA[e,f]	Buffer (0.1 M, pH 8.5)	−8.56

[a]CCA: Concentration of 1 mg/ml.
[b]CPC: Concentration of 25 mg/ml.
[c]EP-CCA: CCA was covalently immobilized on EP.
[d]EP-PEI-CCA: CCA was ionically adsorbed onto PEI-EP.
eEP-EDA-CCA: CCA was ionically adsorbed onto EP-EDA.
[f]The CCA loading amount in the immobilization was 100 unit enzyme per gram support.

After immobilization of the negatively charged CCA, all three supports carried negative charges. Although the binding of proteins on the carrier surface is supposed to cover the particle, the groups on the particle surface still impact the zeta potential of the final preparation. Besides the material and support features, the enzyme loading, and even contributions of part of the protein surface not involved in the immobilization (which depends on enzyme orientation) might affect the zeta potential (Ryan and O'Fágáin 2007; Serra et al. 2013). Due to the polycationic PEI coating on the support surface, the EP-PEI preparation also had the highest zeta potential after immobilization, followed by EP-EDA. The difference in zeta potential of these supports might affect the enzymatic properties of their CCA preparations in some way (Meder et al. 2013; Honary and Zahir 2013).

Immobilization of CCA on different supports

As shown in Figure 1A, the immobilization yield of CCA on the EP, EP-EDA, and EP-PEI supports was nearly 100% when the enzyme loading was low, but decreased gradually with increasing enzyme loading. The immobilization yield of EP-PEI was lower than EP and a little higher than EP-EDA with higher enzyme loading, while the enzyme activity and apparent activity recovery of EP-PEI were higher than that of EP and EP-EDA. The higher zeta potential of EP-PEI did not result in a higher immobilization yield. This might be because the PEI coating on the support reduces the pore diameter or even blocks the pores, and thus the final available surface area for enzyme absorption on EP-PEI is

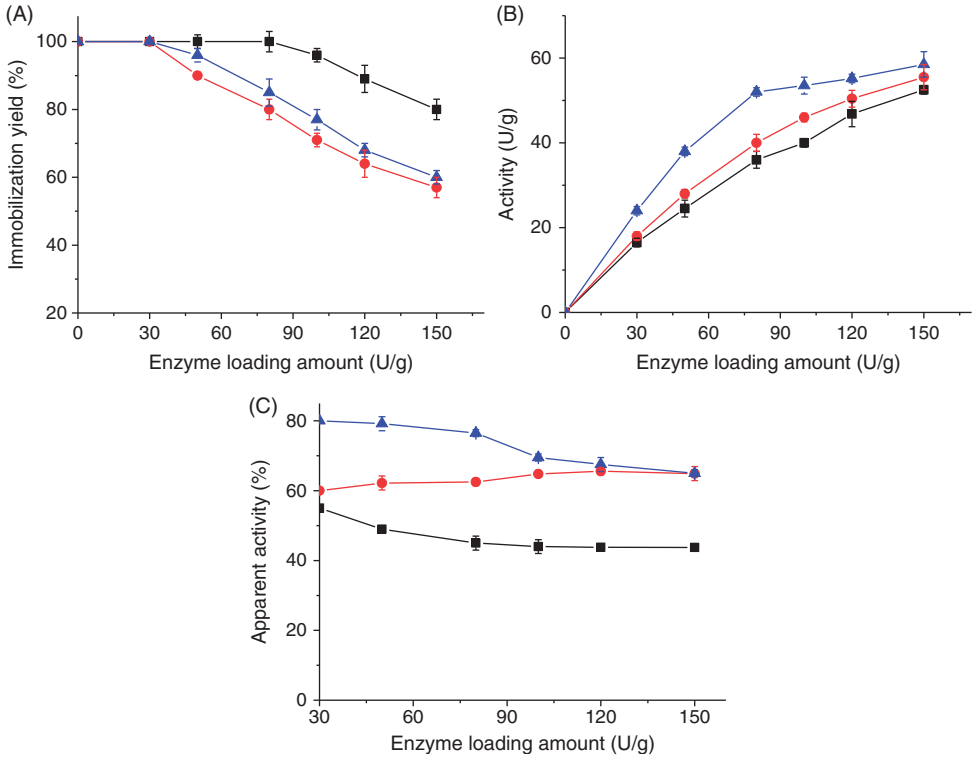

Figure 1. Immobilization of CCA on different supports. (A) Effect of enzyme loading on the immobilization yield. (B) Effect of enzyme loading on the immobilized activity. (C) Effect of enzyme loading on the recovery rate of activity. Symbols: (■) EP; (●) EP-EDA; and (▲) EP-PEI. The immobilization yield is defined as the ratio of the adsorbed enzyme to the initial amount in the solution. The apparent activity recovery is the ratio of the assayed activity of immobilized enzyme to the theoretical activity of adsorbed enzyme.

smaller (Chen et al. 2013; Lin et al. 2013). The EP support had a higher immobilization yield than the ionic supports EP-EDA and EP-PEI. However, the covalent bonds between enzyme and EP support appeared to distort the enzyme structure, leading to a lower activity yield (Mendes et al. 2011; Zhu et al. 2011).

EP-PEI adsorbed more protein than EP-EDA with the same enzyme loading and had a higher zeta potential (Figure 1A, Table 1). This suggests that the polycationic PEI had a more pronounced impact than the small molecule EDA on zeta potential of the immobilized enzymes. Though the PEI grafted supports usually display a relatively low loading capacity for enzymes (Liu et al. 2012), the EP-PEI preparation exhibited the highest apparent activity, and activity recovery (Figure 1A–C). Thus, this cationic polymer coating support seems to be a suitable carrier for CCA immobilization with less activity loss, especially when the purified enzyme with high specific activity was loaded.

Furthermore, the positive zeta potential of EP-PEI and EP-EDA enables them to immobilize the enzyme via electrostatic interaction, which can be achieved in a very short time and at low salt concentration. In contrast, enzyme immobilization on the hydrophobic epoxy support EP requires a high ionic strength buffer to achieve the first step of immobilization, physical adsorption of the enzyme on the support (Barbosa et al. 2013). And the second step of immobilization process, covalent binding of the enzyme to the epoxy support, normally lasts up to 24 h (Zhu et al. 2011; Barbosa et al. 2013).

Thermal stability of different CCA preparations

CCA is a protein with heterogeneous subunits with relatively poor stability during use. As shown in Figure 2, free CCA was not stable when incubated in solution at 45 °C, and its stability correlated with protein concentration suggesting that this multimeric protein tends to dissociate in solution (Bolivar et al. 2006; Rocha-Martin et al. 2009). Thus, stabilization of the CCA quaternary structure is a goal of immobilization, because the dissociation of multimeric enzymes often strongly correlates with enzyme inactivation (Bolivar et al. 2009; Garcia-Galan et al. 2013).

As expected, all the immobilized enzymes showed better stability than their soluble counterparts (Figure 3). Figure 3 shows that the CCA immobilized on EP-PEI had the best stability and its activity declined very slowly at 45 °C. The enzyme immobilized on the cationic support EP-EDA had a half-life of 100 min at 45 °C and pH 8.0, a value much lower

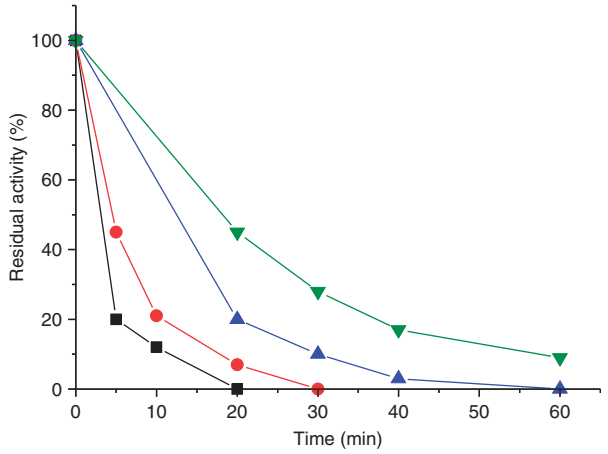

Figure 2. Inactivation courses of free CCA at different concentrations. Symbols: (■) 0.01 U/mL; (●) 0.1 U/mL; (▲) 0.65 U/mL; and (▼) 1 U/mL. Experiments were carried out at 45 °C in 100 mM sodium phosphate buffer pH 8.0.

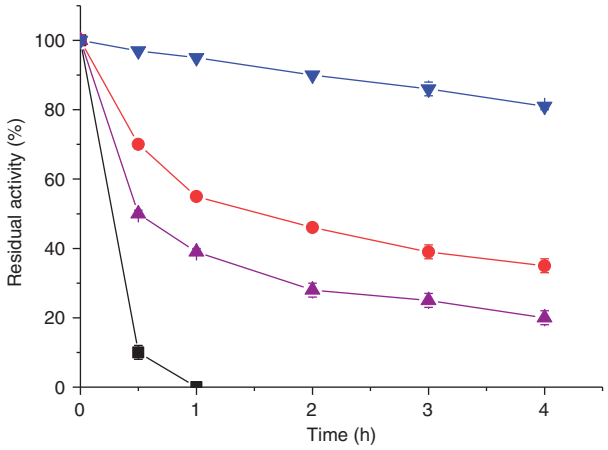

Figure 3. Thermal stability of different CCA preparations. The treatment was performed at pH 8.0 and 45 °C. Symbols: (■) Soluble enzyme; (▲) Enzyme immobilized on EP support; (●) Enzyme immobilized on EP-EDA support; and (▼) Enzyme immobilized on EP-PEI support. The residual activity is expressed as a relative percentage of the original activity.

than on EP-PEI. To quantify the thermal stabilization of immobilized enzyme, the stabilization factor (the ratio of half-life times between immobilisate and soluble enzyme) was calculated. Stabilization factors of enzyme immobilized on EP-PEI and EP-EDA were 35.6-fold and 5.5-fold, respectively.

Although multipoint interactions would occur in the binding of CCA to both EP-EDA and EP-PEI via ionic exchange, the interactions between the enzyme and the EP-EDA support would be relative weak on the "plane" surface (Pessela et al. 2003; Filho et al. 2008). However, the EP-PEI support was provided a flexible cationic polymer coated surface, allowing a greater percentage of the protein surface to interact with the support via a "tridimensional adsorption" (Torres et al. 2002, 2006). Thus, the EP-PEI support is able to establish a non-distorting and very strong ionic interaction with the protein (Torres et al. 2005).

It was shown that the CCA covalently immobilized on the epoxy support had a low thermostabilization factor of 1.5-fold compared with the free enzyme, a value even lower than that of EP-EDA immobilisate (Figure 3). Immobilisates on epoxy support have been reported to have improved stability when immobilization is performed at a higher pH value or by blocking the remaining epoxy groups with highly hydrophilic compounds (Mateo et al. 2002; Barbosa et al. 2013). To reduce the hydrophobicity of the support surface, the remaining epoxy groups of the support were blocked with 3 M glycine. This derivative had unchanged activity but a 17% increased thermal stability. The EP-CCA preparation was also treated at alkaline pH value (pH 10) to produce further covalent binding between enzyme and support. However, the activity of derivative decreased by 23% although the thermal stability slightly increased by 14%. It seems that these protocols for stabilization of the enzyme immobilized on epoxy support, which are effective for a lot of enzymes (Mateo et al. 2000; Mateo et al. 2002), were less effective for CCA.

Since the ε-amino group of lysine residues on the protein surface plays an important role in covalent binding of the enzyme on epoxy or aldehyde activated supports, the number of lysines is crucial for a multi-point covalent binding (Serra et al. 2013). There are nine lysine residues in CCA but only two of them are located on the larger β-subunit, resulting in weak stabilization of the whole enzyme structure (Luo et al. 2014). The very limited and uneven distribution of surface lysine residues might explain why the epoxy support cannot produce a very stable immobilized CCA. Previously published results also suggest that increasing enzyme stability via a few strong covalent bonds is not as good as tridimensional multi-point ionic adsorption (Torres et al. 2005). In our recent work, the covalently immobilized enzyme could be further stabilized by cross-linking with amines (e.g., PEI), which is somewhat similar to stabilization of the tridimensional structure by the cationic polymer PEI in this work (He et al. 2015). However, the covalent immobilization was irreversible so the support could not be reused.

Desorption of enzymes from different ionic supports

Uncontrolled desorption of an enzyme from its support during use is a disadvantage of immobilization via physical adsorption (Filho et al. 2008; López-Gallego et al. 2012). To investigate this, the

enzyme immobilized on EP-PEI and EP-EDA supports was incubated at different salt concentrations to assess the interaction strength between the enzyme and the support. Enzymes on both the EP-PEI and the EP-EDA desorbed gradually from the support when the ionic strength increased. However, the residual activity of EP-PEI immobilized CCA was higher than CCA immobilized on EP-EDA in all the solutions with different ionic strength (Figure 4). Around 70% of CCA remained on EP-PEI when treated with 1 M NaCl solution. The results indicated that the enzyme was more strongly adsorbed on EP-PEI, and the flexible grafted PEI polymer is thought to play an important role. The PEI grafted supports provide a three dimensional surface for interaction with enzymes via multipoint ionic adsorption, where the enzymes may penetrate in the polymeric bed formed by flexible long chains of PEI (Torres et al. 2006). Thus, the adsorbed enzymes are less likely to release from the support during application.

Kinetic parameters of the CCA preparations

The kinetic parameters of CPC hydrolysis were determined for both the free and immobilized CCAs (Table 2). The Lineweaver-Burk plot was linear, indicating that the kinetics of the free and immobilized CCA followed the Michaelis-Menten model.

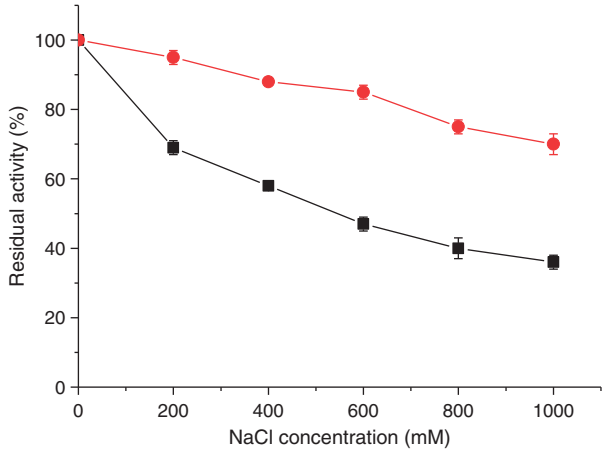

Figure 4. Adsorption–desorption of a protein from different ionic supports. Symbols: (■) EP-EDA and (●) EP-PEI. The residual activity is expressed as a relative percentage of the original activity.

Table 2. Kinetic constants of free CCA and immobilized CCA.

	EP-PEI-CCA	EP-EDA-CCA	EP-CCA	Free CCA
K_m(mM)	22	30	46	43
V_{max} (μmol/min/mg)	6.2	6.0	5.8	15
V_{max}/K_m	0.28	0.2	0.13	0.35

Generally, Michaelis constant K_m is used to evaluate the enzyme-substrate affinity, and a lower K_m value means a higher affinity of substrate to the enzyme. The CCA immobilized on EP showed a slightly higher K_m value than the soluble enzyme. Compared with free enzyme, a large fraction of the immobilized enzyme locates in the pores of the supports, which might decrease the enzyme-substrate binding due to mass transfer limitations and result in an increase in K_m (Shibasaki-Kitakawa et al. 1998). However, the K_m values of CCA immobilized on EP-PEI and EP-EDA were even lower than that of the free enzyme, indicating that a higher substrate affinity was obtained after the immobilization on the cationic supports. Among the three immobilized enzymes, the EP-PEI immobilized enzyme had the smallest K_m value, followed by EP-EDA-CCA, with the K_m values reflecting the zeta potentials of the corresponding supports and their enzyme derivatives.

In multiphase systems with immobilized enzymes, biocatalysis depends on the mobility and partition of substrates (Cantone et al. 2013). The zeta potential of the substrate and enzyme preparations affect the diffusion of substrate from the solution to the microenvironment of immobilized CCA, and the type and content of groups on the support are often important for solute adsorption (Meder et al. 2013). Therefore, the lower K_m values for CCA immobilized on the cationic supports (EP-PEI and EP-EDA) than that of the free enzyme might be ascribed to the increased localized concentration of the negative charged substrate (CPC) near the immobilized enzymes (Meder et al. 2013).

The immobilized enzymes displayed lower V_{max} (maximal initial reaction velocity) and V_{max}/K_m values in comparison to the free counterparts possibly due to enzyme conformational changes induced by the support. EP-PEI-CCA had a larger V_{max} than the enzyme derivatives of EP-EDA and EP-CCA. In addition, the content of amino groups on the support might affect the solutes adsorption, leading to an easier access of the substrate to the enzymes on EP-PEI.

Regeneration and reuse of EP-PEI

Reuse from the supportability of the support is an advantage of reversible immobilization, and it requires the attached enzyme to be desorbed under certain conditions. Increasing the ionic strength or changing the pH of the solution weakens the interactions between the protein and the ionic exchanger (Torres et al. 2002). As shown in Figure 5, CCA was almost completely desorbed from the EP-PEI by using a high concentration of NaCl (e.g., 2 M) at low

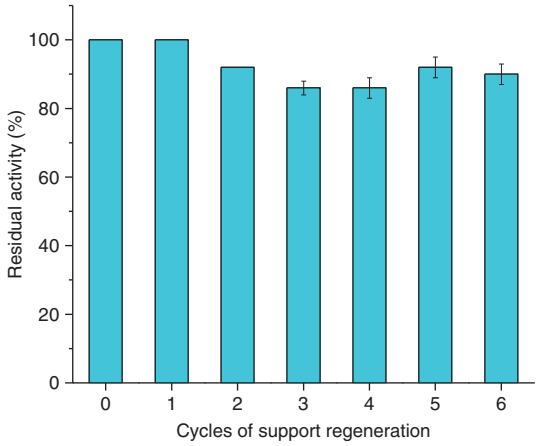

Figure 5. Regeneration and reusability of the EP-PEI support. The residual activity is expressed as a relative percentage of the original activity.

pH value (pH 3.0). The support could be reused for several cycles to immobilize the enzyme with high activity yield.

For biocatalysts applied in industry, the costs of immobilization depend heavily on the carrier, which is often expensive (Cantone et al. 2013). Certainly, the reusability of the EP-PEI support can make biocatalytic process of CCA more cost-effective and economically feasible.

Conclusions

The characteristics of immobilized CCA obtained using EP, EP-PEI, and EP-EDA supports have been compared. The results showed that the best specific activity and thermal stability of immobilized CCA was obtained by physically adsorbing CCA on the EP-PEI support, suggesting that EP-PEI is a very suitable support for reversible but strong and non-distorting ionic protein adsorption, especially for the immobilization of enzymes like CCA, which possess very few surface lysine residues and are difficult to stabilize via traditional covalent immobilization. The reusability of the EP-PEI support significantly reduces the cost of the biocatalyst preparation. In this work, zeta potential was introduced to evaluate the process of enzyme immobilization and the properties of enzyme preparations. Some characteristics, like activity recovery in the immobilization process and the Michaelis constant K_m of immobilized enzymes, were essentially coincident with the zeta potentials of the corresponding supports and enzyme preparations. The results of this work suggest that the zeta potential is a useful indicator of the characteristics of immobilized enzymes and could be helpful in selecting appropriate carriers, especially for the enzyme immobilization on ionic exchange supports.

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