Oriented and selective Enzyme Immobilization On Functionalized Silica Carrier Using the Cationic Binding Module Z_{basic2}: Design of a Heterogeneous D-Amino Acid Oxidase Catalyst on Porous Glass

Juan M. Bolivar and Bernd Nidetzky*

Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

* Corresponding author
E-mail: bernd.nidetzky@tugraz.at; Phone: +43 316 873 8400; Fax: +43 316 873 8434

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Abbreviations: DAO; D-amino acid oxidase; *Tv*DAO; DAO from *Trigonopsis variabilis*

Abstract. D-Amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAO) is usually applied as industrial immobilizate offering enhanced stability under bubble aeration compared with high instability of free enzyme against gas-liquid interfaces. We herein present an "Immobilization by Design" approach that exploits engineered charge complementarity between enzyme and carrier to optimize key features of the immobilization of *Tv*DAO. A fusion protein between *Tv*DAO and the positively charged module Z_{basic2} was generated, and a corresponding oppositely charged carrier was obtained by derivatization of mesoporous glass with 3-(trihydroxysilyl)-1-propane-sulfonic acid. Using 250 mM NaCl for charge screening at pH 7.0, the Z_{basic2} fusion of *Tv*DAO was immobilized directly from *E. coli* cell extract with almost absolute selectivity and full retention of catalytic effectiveness of the isolated enzyme in solution. Attachment of the homodimeric enzyme to the carrier was quasi-permanent in low-salt buffer but fully reversible upon elution with 5 M NaCl. Immobilized *Tv*DAO was not sensitive to bubble aeration and received substantial (≥ tenfold) stabilization of the activity at 45 °C as compared to free enzyme, suggesting immobilization via multisubunit oriented interaction of enzyme with the insoluble carrier. The Z_{basic2} enzyme immobilizate was demonstrated to serve as re-usable heterogeneous catalyst for D-amino acid oxidation. Z_{basic2}-mediated binding on a sulfonic acid group-containing glass carrier constitutes a general strategy of enzyme immobilization supporting transition from case-specific empirical development to more rational design.

Key words: Enzyme immobilization; cationic binding module; Z_{basic2}; cation exchange; charge complementarity; one-step purification-immobilization; random and oriented binding

INTRODUCTION

D-Amino acid oxidase from the yeast *Trigonopsis variabilis* (*Tv*DAO) is used industrially for the synthesis of pharmaceutical intermediates and fine chemicals (Pilone and Pollegioni 2002; Pollegioni et al. 2008). A widely known application of *Tv*DAO is in the process for production of 7-amino-cephalosporanic acid where the initial oxidation of the cephalosporin C substrate by *Tv*DAO is a key step (Riethorst and Reichert 1999; Volpato et al. 2010). Another commercialized use of *Tv*DAO is in the synthesis of enantiopure non-natural L-amino acids. A dynamic kinetic resolution process has been implemented where *Tv*DAO oxidizes the unwanted D-antipode from the racemic amino acid substrate to an intermediary imine that is non-specifically reduced with a chemical reagent (Hollmann et al. 2011; Turner 2011). After several cycles of oxidation and reduction, the L-configured product is obtained in high purity.

The catalytic cycle of *Tv*DAO involves reduction of O_2 to H_2O_2. The O_2 required in the biocatalytic conversion is usually supplied by bubble aeration, and agitation is furthermore used to entrain the air (Pilone and Pollegioni 2002). However, *Tv*DAO in its soluble form is highly sensitive to exposure to an air-liquid interface (Dib and Nidetzky, 2008), necessitating the use of alternative forms of the catalyst that show enhanced resistance to the operating conditions of an aerated bioreactor. *Tv*DAO is a functional homodimer, and dissociation of its subunits probably presents the major cause for

enzyme inactivation (Fernández-Lafuente et al. 1999; Betancor et al. 2003; Fernandez-Lafuente 2009). Whole-cell catalysts were derived from the native *T. variabilis* or from heterologous hosts expressing the *Tv*DAO gene (*E. coli*, *S. cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*) (Abad et al. 2011; Brodelius et al. 1981; Isoai et al. 2002; Tan et al. 2007; Vicenzi and Hansen 1993). The enzyme naturally encapsulated by the cell used for its production is substantially more stable than the corresponding isolated preparation of the oxidase (Abad et al. 2010; Dib et al. 2007). *Tv*DAO artificially entrapped in polymeric microcapsules also displays improved stability (Nahalka et al. 2008). In an alternative approach, immobilization of *Tv*DAO on porous carriers was considered as means of enhancing the total turnover number of the enzyme in the process (Betancor et al. 2003; Bianchi 1995; Dey et al. 1991; López-Gallego et al. 2005a; López-Gallego et al. 2005b; Riethorst and Reichert 1999; Zheng et al. 2006). When compared to whole cells, enzyme immobilizates generally offer the important advantages of facile re-use and clean separation from the liquid phase containing the product (Riethorst and Reichert 1999; Volpato et al. 2010). On the downside, however, the immobilization generates extra costs (Tufvesson et al. 2010; Tufvesson et al. 2011) and in the case of *Tv*DAO, an important issue of the reported immobilization procedures is serious loss (≥ 50%) of the original enzyme activity (Betancor et al. 2003; López-Gallego et al. 2005a; López-Gallego et al. 2005b; Dib and Nidetzky 2008). By today, *Tv*DAO immobilization remains technically difficult and constitutes an economically weak point in the different biocatalytic processes developed with this enzyme (Pilone and Pollegioni 2002; Pollegioni et al. 2008). DAAO is also

applied in other biotechnological applications (Pollegioni and Molla 2011) as biosensors; an optimal immobilization approach is highly desired in this case.

We have therefore chosen *Tv*DAO as a technologically relevant model for the development of what constitutes according to our opinion a generally applicable approach of enzyme immobilization. This approach builds on an engineered enzyme-to-carrier charge complementarity that is sufficiently strong to allow for a stable and selective, yet non-covalent and thus practically reversible attachment of the target enzyme onto a suitable insoluble support. By eliminating the need to work with (partially) purified enzyme, this being a major cost-contributing factor in most conventional immobilization procedures, a high binding selectivity gives a definite advantage (Garcia-Galan et al. 2011). Even though a broad spread of procedures coupling purification and immobilization are previously described, not many of them fulfill the requirement of a general immobilization strategy (Garcia-Galan et al. 2011; Hernandez and Fernandez-Lafuente 2011). Reversibility of the immobilization makes it possible to regenerate the carrier for repeated use, which is a potential dictate of economic considerations (Garcia-Galan et al. 2011). However, there is the problem that the electrostatic properties of enzymes are not commonly suitable to tether them in an active conformation stably on the surface of a charged carrier (Hanefeld et al. 2009). Moreover, each enzyme is unique and thus is becomes practically impossible to exploit the native protein structure for development of a selective and generally useful procedure of immobilization (Hanefeld et al. 2009).

As a solution we have proposed to fuse the target enzyme to the cationic binding module Z_{basic2}, which is an engineered arginine-rich variant of the Z domain, a 58 amino acid (7 kDa) three-helix bundle obtained from the B domain of staphylococcal protein A (Gräslund et al. 2002; Graslund et al. 2000; Hedhammar and Hober 2007). The extra positive charge acquired by the Z_{basic2} enzyme provides a driving force enabling a strong attachment of the protein and a selective immobilization, since most of proteins presents a low pI to be attached on an anionic support at neutral pH (Wiesbauer et al. 2011). Additionally, the large charge asymmetry present in a Z_{basic2} enzyme steers the preferred orientation(s) of the protein upon binding such that interaction with the carrier surface will primarily be mediated by the Z_{basic2} module (Wiesbauer et al. 2011). Unlike charged peptide tags that have no defined structure and are therefore unpredictable concerning their effect on protein binding to supports, the Z_{basic2} module can be viewed a largely independent unit of structure and function in the respective fusion protein. In addition to oriented immobilization, this would mean the module is probably protecting the enzyme of undesired secondary interactions with the carrier surface (Scheme 2). Because immobilization of Z_{basic2} enzymes is built on a common principle, lessons learned with one enzyme should be transferable at large to others without having to go through the same procedure of mostly empirical optimizations for each enzyme anew. The process development would become better predictable and also substantially accelerated at the level of the enzyme immobilization. The results for *Tv*DAO show that immobilization of Z_{basic2} enzyme on porous glass derivatized with sulfonic acid surface groups presents a powerful and highly modular opportunity to obtain a heterogeneous biocatalyst in effective yield, high specific activity, and stabilized form. The proposed immobilization

method is potentially broad in scope not only with respect to the enzyme used but also concerning the type of carrier applied. In particular, it opens up the possibility to utilize a variety of mesoporous silica-derived supports for enzyme immobilization (Hartmann 2005; Hartmann and Jung 2010; Hoffmann et al. 2006; Hudson et al. 2008; Moelans et al. 2005). Despite very promising material properties for technological use, the known silica-based carriers share the drawback that in native form, their surface is generally unsuitable for binding of enzymes. Therefore, development of silica-supported enzyme catalysts necessitates tailoring of the carrier surface properties through case-specific optimization (Hartmann and Jung 2010; Moelans et al. 2005; Yiu and Wright 2005). It is thus vitally important that the herein used chemistry of glass surface functionalization is fully compatible with silica-derived material in general, including diatomaceous earth and various kinds of zeolithes (Corma et al. 2002; Mitchell and Pérez-Ramírez 2011). Their use range from glass chips in microfluidic analytics to enzyme carriers for industrial biotransformations, spanning 10 or more orders of magnitude in the applied process scale. The case of *Tv*DAO is used here to illustrate the clear advantages of the proposed innovative approach of oriented non-covalent enzyme immobilization.

MATERIALS AND METHODS

Materials

TRISOPERL® controlled pore glass (CPG) beads were obtained from VitraBio GmbH (Steinach, Germany) with the following specifications: 50-100 µm diameter, 118.04 nm pore size, 1225.46 mm^3/g pore volume, and 44.04 m^2/g specific surface area. 3-Aminopropyl-triehoxysilane (APTES), (3-glycidoxypropyl) trimethoxysilane (GPMES), and 3-(trihidroxysilyl)-1-propane-sulfonic acid (SPTHS) were from Gelest Inc.

(Morrisville, PA, USA). All other materials used were of reagent grade or better quality. The Z_{basic2} fusion of *Tv*DAO (Z_{basic2}_*Tv*DAO) was prepared as described in a recent publication (Wiesbauer et al. 2011). *Tv*DAO harboring Strep-tag II (Strep_ *Tv*DAO) was used as reference (Dib et al. 2007; Wiesbauer et al. 2011). If not mentioned otherwise, *E. coli* cell extract containing Z_{basic2}_*Tv*DAO or Strep_ *Tv*DAO was used. Purified enzyme standards were prepared by reported protocols (Dib et al. 2007; Wiesbauer et al. 2011).

Carrier Surface Functionalization

Derivatization of CPG with APTES, GPMES, and SPTHS was performed according to a reported procedure and yielded CPG activated with amino (CPG-amino) (Howarter and Youngblood 2006), epoxy (CPG-epoxy) (Porsch 1993), and sulfonate groups (CPG-sulfonate) (Athens et al. 2009), respectively. CPG-epoxy was further modified with L-Asp to introduce carboxylate surface groups (CPG-carboxylate) (Batalla et al. 2008). CPG-amino was derivatized with glutaraldehyde and then further modified with glycine or amino-sulfonic acid to introduce a carboxylate or sulfonate group at the end of a four-carbon spacer arm (CPG-C_4-carboxylate; CPG-C_4-sulfonate). The full protocols of the CPG derivatizations used are given in the Supporting Information.

Assays

Protein was measured with the Bradford dye-binding assay calibrated against BSA. Activity of *Tv*DAO was measured using a peroxidase-coupled assay employing D-Met or D-Ala, each at 10 mM, as substrate (Maeda-Yorita et al. 1995; Wiesbauer et al. 2011). One U of activity corresponds to 1 µmol of H_2O_2 produced/min under the conditions used. The specific activity of cell extract containing Z_{basic2}_*Tv*DAO was typically about 2

U/mg while the purified Z_{basic2}_*Tv*DAO had a specific activity of 71 U/mg using D-Met as substrate.

Immobilization

E. coli cell extract containing of Z_{basic2}_*Tv*DAO or Strep_*Tv*DAO was resuspended in 50 mM potassium phosphate buffer at pH 7. The total protein content varied between 10- 50 mg/mL and the activity of DAO between 20-100 units/mL. About 100 mg of washed activated CPG particles was incubated at 25 °C under gentle agitation with 1 mL of this protein mixture. To determine the progress of the immobilization, samples were withdrawn at certain times, and protein and enzyme activity remaining in the supernatant were measured using the assays described above. At some mentioned cases the concentration of potassium phosphate buffer used was 10 mM and a variable concentration of NaCl (0-1 M) was added to the protein mixture. Unless mentioned, all enzyme immobilizates were washed with potassium phosphate buffer (50 mM, pH 7.0, 250 mM NaCl) prior to any further use. Immobilization was typically performed in a single loading experiment. However, to examine the binding capacity of the different carriers used, we carried out several rounds of immobilization under the conditions described above, using an incubation time of 1 h and an intermediate washing step with loading buffer in each round. SDS PAGE (See supporting information) was used to complement protein and activity measurements as probes of binding to and elution from the carrier. Immobilized activity/protein was calculated by the difference between the offered and the residual activity/protein contained in the supernatant during immobilization; immobilization yield express the percentage of activity/protein bound divided by the activity/protein offered to the carrier.

Elution of Z_{basic2}_*Tv*DAO From CPG-carboxylate and CPG-sulfonate

Immobilized Z_{basic2}_*Tv*DAO was incubated in 10 mM potassium phosphate buffer at pH 7.0 and 25 ºC. About 1 g CPG immobilizate/mL containing 200 U of activity, corresponding to approximately 7 mg of protein, was used. The buffer contained different concentrations of NaCl. The suspension was incubated under gentle stirring for 30 min. Enzyme activity and protein concentration in the supernatant were determined.

Catalytic Effectiveness of Immobilized Z_{basic2}_*Tv*DAO

The catalytic effectiveness of the enzyme immobilizate (η) is the ratio of the *observable* activity of the immobilized enzyme ($V_{observable}$; U/$g_{carrier}$) compared to the *theoretical* activity of the immobilized enzyme ($V_{theoretical}$) that would result if the immobilized enzyme had the same activity as the free enzyme. $V_{theroretical}$ is therefore calculated from the difference in activity in solution before and after the immobilization, and it is also expressed as U/$g_{carrier}$. For determination of η, enzyme immobilizates were used that showed variable $V_{theoretical}$ in the range 1 – 500 U/$g_{carrier}$. For determination of the corresponding $V_{observable}$, reactions were performed under conditions of the peroxidase-coupled assay. Two mL solution of 10 mM D-Met in 100 mM potassium phosphate buffer, pH 8.0, supplemented with 4-aminoantipyrine (1.5 mM), N,N-dimethylaniline (0.04 % v/v) and horse radish peroxidase (10 µg/mL) was incubated in the presence of immobilizate at 30 °C and under magnetic stirring at 300 rpm. Samples of about 200 µL were taken at certain times over 30 min, absorbance at 565 nm was measured quickly, and the sample was returned to the original mixture. The amount of immobilizate added was varied to adjust a suitable reaction rate of about 15 milli-absorbance units/min.

Stability of Immobilized Z_{basic2}_*Tv*DAO

The stability of free and immobilized Z_{basic2}_*Tv*DAO was compared under temperature stress conditions of 45 °C at pH 8.0. Enzyme concentrations used during the incubation were varied as indicated under Results. Activity remaining in solution (free enzyme) or bound to the carrier (immobilizate) was measured in samples taken over time. Stability under conditions of bubble aeration (Stirring at 1000 rpm was used to entrain air) was measured at 25 °C in a magnetically stirred flask of 28 mm diameter and 114 mm height. The liquid volume was 15 mL. The enzyme solution consisted of 50 mM potassium phosphate buffer, pH 8.0, and contained 0.05 – 0.25 U/mL of activity. Samples were taken over time for activity measurement. Controls were carried out identically without stirring.

Enzymatic Oxidation of D-Ala With Repeated Re-use of Immobilized Enzyme

The above-described stirred flask was used. Eight mL of a solution containing D-Ala in 50 mM potassium phosphate buffer (pH 8.0) were used. The reaction mix was supplemented with bovine catalase (0.01 mg/mL). About 40 mg of Z_{basic2}_*Tv*DAO immobilizate on CPG-sulfonate (54 $U/g_{carrier}$) were added. Under the conditions of surface aeration used (1000 rpm stirrer speed), the oxygen transfer coefficient was determined as 1.5/min. It was ensured by proper choice of enzyme loading that supply of air oxygen was not rate-limiting for the enzymatic conversion overall. At the times indicated, samples were withdrawn, and the concentrations of D-Ala and pyruvate were determined, as previously described (Dib et al. 2007), and an enzymatic assay based on lactate dehydrogenase (Valero and García-Carmona 1996). The enzyme immobilizate

was recovered by sedimentation after the reaction had been completed, and it was used again for two new rounds of conversion.

Statistical Treatment of the Data Presented in the Figures The results show the average value of several experiments. Error bars represent the standard deviation of three replicate experiments.

RESULTS AND DISCUSSION

Selection of a Matched Carrier for Immobilization of Z_{basic2} Enzyme

Silica-based materials constitute an emerging class of supports for immobilization of proteins (Moelans et al. 2005; Yiu and Wright 2005; Hoffmann et al. 2006; Hudson et al. 2008; Hartmann and Jung 2010). We have chosen CPG as model of a silica-derived carrier, considering that the internal structure of CPG is very well defined and the size of the available pores (118.04 nm diameter) should enable relatively unrestricted access of *Tv*DAO on the entire available surface area. Note: It was estimated from the crystal structure of the homologous DAO from *Rhodotorula gracilis* that the enzyme has a dimension of about 5.6 × 14.8 × 7.1 nm. Therefore, the use of CPG made sure that study of the interaction between Z_{basic2}_*Tv*DAO and different silica surfaces was not compromised by other structural peculiarities of the applied carrier.

Irrespective of the carrier used in a standard immobilization experiment (50 mM potassium phosphate buffer, pH 7.0), binding of Z_{basic2}_*Tv*DAO was about 90% complete in 30 min, and no change in the concentration of total protein and oxidase activity in the supernatant was observed after 1 h. However, when analyzing the immobilization at this apparent equilibrium, the role of the surface functionalization was revealed distinctly.

CPG-C_4-carboxylate and CPG-C_4-sulfonate carriers showed a highly non-specific binding where about 50% of total offered protein and enzyme activity was pulled down from the supernatant. The calculated specific activity of immobilized Z_{basic2}_*Tv*DAO was therefore not changed much (≤ twofold) as compared to the original specific activity in the cell extract. The CPG-carboxylate carrier gave a by far more specific binding that could be expressed as a roughly eightfold increase in specific activity. However, just about 50% of total offered activity was bound to CPG-carboxylate. CPG-sulfonate clearly stood out of the different carriers examined because it bound all of the offered enzyme activity while binding just about 8% of total protein at the same time. The specific activity of bound Z_{basic2}_*Tv*DAO was 30 U/mg corresponding to 42% of the specific activity of the purified enzyme (Supporting Figure S1). The superior performance of CPG-sulfonate and CPG-carboxylate as compared to the corresponding spacer arm-containing carriers, CPG-C_4-sulfonate and CPG-C_4-carboxylate, could be explained by a less efficient surface modification due to the instability of glutaraldehyde and the instability of generated bond with amino group (Migneault et al. 2004; Walt and Agayn 1994). In addition, the complexity of the spacer arm introduced (mixed ionic exchanger with hydrophobic moiety) could hinder the selectivity of the protein immobilization. For this reason, CPG-C_4-sulfonate and CPG-C_4-carboxylate carriers were not further pursued.

Optimized Immobilization of Z_{basic2} Enzyme on CPG-sulfonate

To examine the influence of ionic strength on the selectivity of binding of Z_{basic2}_*Tv*DAO to CPG-sulfonate, we decreased the phosphate buffer strength to 10 mM and added NaCl in different concentrations (0 – 250 mM) to the reaction mixture. While all of the offered enzyme activity was bound to CPG-sulfonate irrespective of the absence or presence of

NaCl, the specific activity of bound Z_{basic2}_*Tv*DAO was enhanced in clear dependence of the salt concentration, increasing from 10 U/mg when no NaCl was added to 45 U/mg at 250 mM NaCl (Supporting Figure S2). Use of even higher concentrations of NaCl up to 500 mM were not effective in further improving the specific oxidase activity and caused problems due to precipitate formation in the cell extract. It was shown in control experiments that Strep_*Tv*DAO was hardly immobilized (< 10% of total activity) on CPG-sulfonate both in the absence or presence of NaCl, suggesting that the enzyme part of the Z_{basic2}_*Tv*DAO fusion protein should have no detectable affinity for binding to the anionic support; between 10-20 % of protein was immobilized during the time span of experiment, constituting a percentage of protein usually attach at pH 7 to a cationic exchanger using a cell extract (Fuentes et al. 2004). Attachment of Z_{basic2}_*Tv*DAO to CPG-sulfonate would thus occur exclusively via the cationic binding module, leading to the presumed oriented immobilization of the enzyme. The enhanced selectivity of the binding was enabled by distinctive properties of Z_{basic2} module, the richness in net positive charges distributed on the surface of the domain (Hedhammar and Hober 2007) enables a multipoint cationic exchange even in the hindering conditions caused by increased ionic strength.

When after immobilization of Z_{basic2}_*Tv*DAO on CPG-sulfonate or CPG-carboxylate the enzyme immobilizate was resuspended in loading buffer (50 mM potassium phosphate, pH 7.0), no elution of enzyme activity to the supernatant was observed over a period of 24 h. This suggested that Z_{basic2}_*Tv*DAO was very stably bound to both carriers. To further examine the apparent relative strength of binding to CPG-sulfonate and CPG-carboxylate, we analyzed the release of protein and enzyme activity

from either carrier upon addition of NaCl. Figure 1 shows that in the presence of 1 M NaCl, all of the protein and most of the activity bound to CPG-carboxylate had become washed off. By way of comparison, Z_{basic2}_*Tv*DAO bound to CPG-sulfonate was eluted only after applying 5 M NaCl, and several cycles of elution were needed to recover ≥ 80% of the immobilized enzyme activity. Change of pH to a value of 9.0 facilitated recovery of Z_{basic2}_*Tv*DAO from CPG-sulfonate, yielding almost pure enzyme in a single elution step when applying 5 M NaCl (Figure S3). Moreover, the carrier was recovered in a fully reusable quality through this procedure. Figure 1 also shows that protein became released from CPG-sulfonate at a by far lower salt concentration (≤ 2 M) than was needed to release the activity. These results portray an unusually strong type of reversible binding of Z_{basic2}_*Tv*DAO to CPG-sulfonate that offers use of the enzyme immobilizate under a wide range of ionic conditions, without risk of elution of the activity. They also imply that CPG-sulfonate clearly surpassed CPG-carboxylate in terms of strength of binding of Z_{basic2}_*Tv*DAO, arguably reflecting differences in the cationic exchange properties of the functional groups used (CH_3SO_3H: strong, $pK_a = -2.6$; $-CO_2H$: weak, $pK_a = 2.10$ for α-carbon carboxylic group, $pK_a = 3.86$ for side chain). They furthermore reveal that binding to CPG-sulfonate was highly specific for Z_{basic2}_*Tv*DAO.

Considering the relative ease of elution of non-specifically bound protein as compared to elution of Z_{basic2}_*Tv*DAO from CPG-sulfonate (Figure 1), we tested the effect of washing the CPG-sulfonate immobilizate with NaCl solution on the specific activity of the bound enzyme. Starting from an initial specific activity of about 30 U/mg obtained through the standard immobilization procedure, it was possible to increase the specific activity to 45 U/mg and 62 U/mg using a single wash of the immobilizate with

0.5 and 2.0 M NaCl, respectively. Analysis by SDS PAGE of the protein bound to the carrier (see the Supporting Information) confirms the gradual increase in purity of immobilized Z_{basic2}_*Tv*DAO, reflected by enhancement of specific activity (Figure S3).

In conclusion, therefore, surface functionalization with SPTHS to introduce sulfonate groups was identified as a powerful strategy to prepare glass and by extension, other silica-derived materials, for immobilization of Z_{basic2} enzyme. Charge screening in the presence of 250 mM NaCl can be used to suppress in large part the non-specific binding of proteins from the *E. coli* cell extract. The specific activity of the immobilized Z_{basic2} enzyme may be further enhanced by after washing with NaCl. Removal of Z_{basic2} enzyme for re-use of CPG-sulfonate may be done at a suitably elevated pH using NaCl.

Catalytic Effectiveness of Immobilized Z_{basic2}_*Tv*DAO

CPG-sulfonate immobilizates were prepared that differed in $V_{theroretical}$ between 1.00 and 500 U/$g_{carrier}$. We performed repeated loading experiments under standard immobilization conditions to achieve a $V_{theroretical}$ exceeding 200 U/$g_{carrier}$. It was shown (Figure S4) loadings that the amount of activity and protein bound to CPG-sulfonate increased linearly with each of four loading steps. All of the offered activity (200 U/$g_{carrier}$/step) was immobilized. These results suggest that the binding capacity of CPG-sulfonate for Z_{basic2}_*Tv*DAO should be generally useful for practical application, even though we did not attempt to fully saturate the carrier with target enzyme. We show below (Figure 2) that CPG-sulfonate would become greatly "overloaded" with activity at $V_{theroretical}$ values greater than 500 U/$g_{carrier}$. However, such overloading may be desired in practice, supporting long-term use of the heterogeneous catalyst.

Figure 2 displays a correlation between η and $V_{theroretical}$. At a very low $V_{theroretical}$ of 1.00 U/$g_{carrier}$, immobilized Z_{basic2}_*Tv*DAO shows the same activity as the free enzyme, reflected by η ≈ 1. This result is important because it implies no loss of enzyme activity resulting from the attachment of Z_{basic2}_*Tv*DAO to the CPG-sulfonate carrier. This in turn is consistent with the notion that oriented binding via Z_{basic2} will not interfere with the function of the enzyme at large. The presumed advantages of oriented immobilization in the way promoted by Z_{basic2} is therefore fully realized when Z_{basic2}_*Tv*DAO binds to CPG-sulfonate (Hernandez and Fernandez-Lafuente 2011; Wiesbauer et al. 2011). Not only does this validate the strategy of protein design used but it also supports the use of CPG-sulfonate as matched carrier for Z_{basic2} enzyme immobilization.

When $V_{theroretical}$ was raised gradually, η first dropped very steeply to a value of about 0.4 at 50 – 100 U/$g_{carrier}$ and then decreased in a shallow manner (Figure 2). Considering the results from the repeated loading experiments (Figure S4) that at a $V_{theroretical}$ value of 50 U/$g_{carrier}$ the CPG-sulfonate carrier was massively unsaturated with Z_{basic2}_*Tv*DAO, the observed decrease in η cannot be explained by effects of high surface coverage with target enzyme. It is much more likely that diffusion, in particular that of the O_2 cosubstrate, becomes a limiting factor for $V_{observable}$ as $V_{theroretical}$ increases, hence the loss in η (Buchholz et al. 2005). The biphasic character of the relationship between $V_{theroretical}$ and η may be related to the formation of intraparticle O_2 gradients in the first phase where η drops sharply, and the complete depletion of O_2 at high $V_{theroretical}$ (≥ 100 U/$g_{carrier}$) in the second phase where the decrease of η is relatively shallow. Although interesting, the experiments needed to substantiate interpretation of the η-$V_{theroretical}$ dependence in terms of a reaction-diffusion model was beyond the scope of this work.

Stability of Immobilized Z_{basic2}_*Tv*DAO

Multipoint, typically covalent attachment onto insoluble supports may offer extra stability to the enzyme immobilized, most often explained phenomenologically as the result of a structural rigidification effect (Mateo et al. 2007). Oriented immobilization of a Z_{basic2} enzyme is not expected to provide a similar stabilization due to the absence of direct interactions between the enzyme and the anionic support (Hernandez and Fernandez-Lafuente 2011; Wiesbauer et al. 2011). Considering that stability enhancement in multipoint enzyme immobilizates usually occurs in a complex, a priori unknown relationship with a corresponding loss of catalytic activity, we would argue that in terms of predictability and design of the immobilization there would be a distinct advantage to methods preventing structural distortions in the target enzyme from taking place (Hanefeld et al. 2009; Mateo et al. 2007).

Figure 3A compares time courses of inactivation of Z_{basic2}_*Tv*DAO in solution and bound to CPG-sulfonate under conditions of bubble aeration. While the soluble enzyme was destroyed rapidly, the immobilizate was almost fully stable during the time span of the experiment. The large extra stability recruited by Z_{basic2}_*Tv*DAO from the immobilization on CPG-sulfonate is most likely explainable as protection of the internally bound enzyme against denaturing contacts with air bubble surfaces (Bolivar et al. 2006; Bommarius and Karau 2005; Findrik et al. In Press). Literature shows that *Tv*DAO immobilized conventionally via multipoint covalent attachment to a porous carrier (Sepabeads) was stabilized to a similar degree in comparison to the soluble enzyme as we demonstrated herein for the Z_{basic2}_*Tv*DAO immobilizate (Dib and Nidetzky 2008). Oriented immobilization via Z_{basic2} on a porous support such as CPG-

sulfonate is therefore sufficient to remove the main cause of inactivation of the soluble enzyme under the conditions of biocatalytic processing (Khoronenkova and Tishkov 2008; Pilone and Pollegioni 2002).

Free and immobilized enzymes were exposed to an elevated temperature of 45°C and slightly basic pH. Time courses of inactivation were measured at two initial levels of the enzyme activity that were exactly comparable among the preparations of Z_{basic2}_*Tv*DAO used. Figure 3B shows that soluble Z_{basic2}_*Tv*DAO was inactivated substantially faster at the lower enzyme concentration which is as expected if inactivation of the enzyme that functions as a homodimer involved subunit dissociation as a relevant denaturation step. For the Z_{basic2}_*Tv*DAO immobilizate, by contrast, a similar concentration dependence of the inactivation was lacking completely. Moreover, immobilized Z_{basic2}_*Tv*DAO displayed greater resistance to inactivation at 45 °C than the soluble enzyme at each concentration. Taken together, these findings strongly support the idea that subunit dissociation has a major role in enzyme inactivation (Betancor et al. 2003; Fernandez-Lafuente 2009; Fernández-Lafuente et al. 1999), and that oriented immobilization via Z_{basic2} stabilizes the functional dimmer (Fernandez-Lafuente 2009). It is tempting to speculate therefore that dimer stability in immobilized Z_{basic2}_*Tv*DAO results from a "bivalent" binding mode with the CPG-sulfonate support, involving interactions from both Z_{basic2} modules at a time (Fernandez-Lafuente 2009). Scheme 2.

Biotransformation With Repeated Re-use of Immobilized Z_{basic2}_*Tv*DAO

We used oxidative deamination of D-Ala into pyruvate as model reaction to evaluate the suitability of the Z_{basic2}_*Tv*DAO immobilizate on CPG-sulfonate for application in semi-continuous biotransformations. Figure 4 shows results from an experiment that involved

repeated re-use (by sedimentation) of the immobilized enzyme for three cascaded batch conversions of 50 mM D-Ala. In each round of reaction, substrate was converted fully and pyruvate was obtained in quantitative yield. The space-time yield (~17 mM/h) was also constant across the three conversions and corresponded to about 90% of the theoretically maximum reaction rate. This result indicates that the immobilized enzyme was stable during the reaction and there was no loss of activity to the supernatant by leaching from the carrier. Overall, the performance parameters for immobilized Z_{basic2}_*Tv*DAO are very promising.

CONCLUSIONS

We have developed a generally applicable method for non-covalent oriented immobilization of enzymes on silica supports. Tailored electrostatic complementarity between the positively charged binding module Z_{basic2} and the carrier surface is introduced through a simple one-step functionalization of the silica surface with negatively charged sulfonate groups. The case of *Tv*DAO reveals that Z_{basic2} enzyme binds to the carrier with very high affinity and exquisite selectivity, eliminating the requirement of intermediate purification for enzyme immobilization. Because binding of Z_{basic2} enzyme is readily reversible under high salt conditions, the carrier can be regenerated for repeated use. Immobilized Z_{basic2}_*Tv*DAO is shown to be a fully active, yet substantially stabilized heterogeneous biocatalyst, indicating that the proposed strategy of enzyme immobilization has a clear relevance for bioprocess development. The results provide an important step towards a practically useful enzyme immobilization by design.

Acknowledgements

J.M.B. acknowledges a fellowship (project EX2009-0053) from the Spanish Ministerio de Educacion.

FIGURE CAPTIONS

Scheme 1. Production of L-amino acids based on a chemo-enzymatic process of dynamic kinetic resolution.

Scheme 2. Non-covalent oriented immobilization of Z_{basic2}_*Tv*DAO by electrostatic multi-subunit interaction with CPG-sulfonate. Proteins lacking clustered positive charge on their surface will not bind strongly or may be repelled from the carrier surface. At neutral pH, most of the proteins will have a negative net charge. The shown structure of Z_{basic2}_*Tv*DAO is for illustration.

Figure 1. **Z_{basic2}_*Tv*DAO bound to CPG-carboxylate and CPG-sulfonate is eluted at high salt concentration.** Enzyme activity (continous line) and protein (dotted line) eluted from CPG-carboxylate (open symbols) and CPG-sulfonate (full symbols) are shown.

Figure 2. Catalytic effectiveness of Z_{basic2}_*Tv*DAO immobilized on CPG-sulfonate. See the Materials and methods for details.

Figure 3. Inactivation of Z_{basic2}_*Tv*DAO immobilized on CPG-sulfonate under conditions of bubble aeration (A) and at elevated temperature (B). In panel A, the symbols show soluble Z_{basic2}_*Tv*DAO (0.25 units/mL, full circles; 0.05 units/mL, open circles) and immobilized Z_{basic2}_*Tv*DAO (full diamonds). In panel B, the symbols show soluble Z_{basic2}_*Tv*DAO (0.05 units/mL, full circles; 0.01 units/mL, open circles) and immobilized Z_{basic2}_*Tv*DAO (0.05 units/mL, full diamonds; 0.01 units/mL, open diamonds). The temperature was 45 °C and the pH 8.0. See the Materials and methods for details.

Figure 4. Semi-continuous biocatalytic conversion of 50 mM of D-Ala in pyruvate using re-use of Z_{basic2}_*Tv*DAO immobilized on CPG-sulfonate. See the Materials and methods for details.

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Figure 1

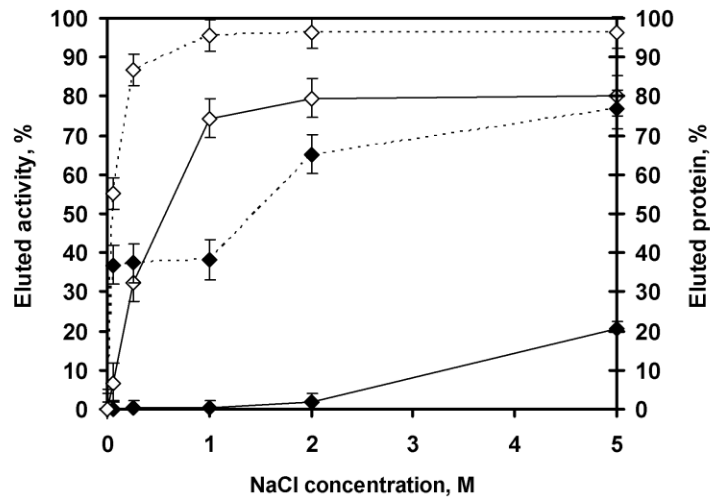


Figure 2

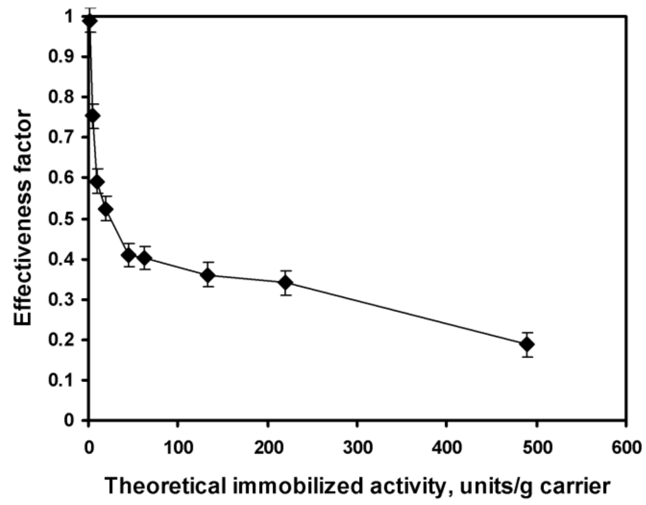
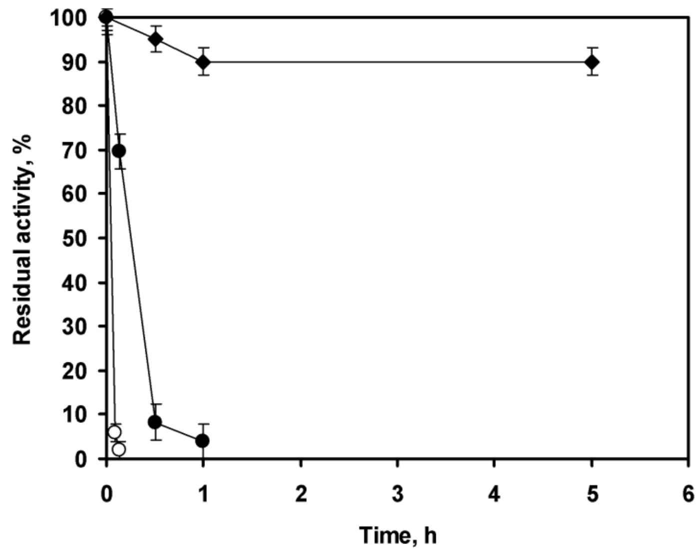


Figure 3A



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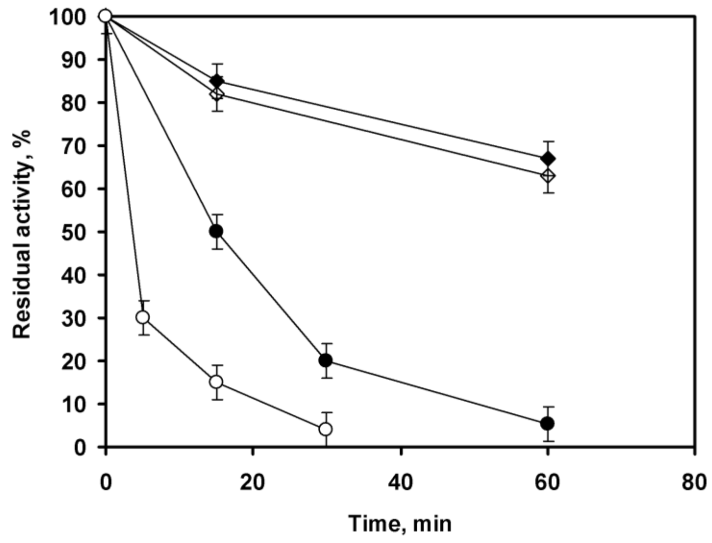
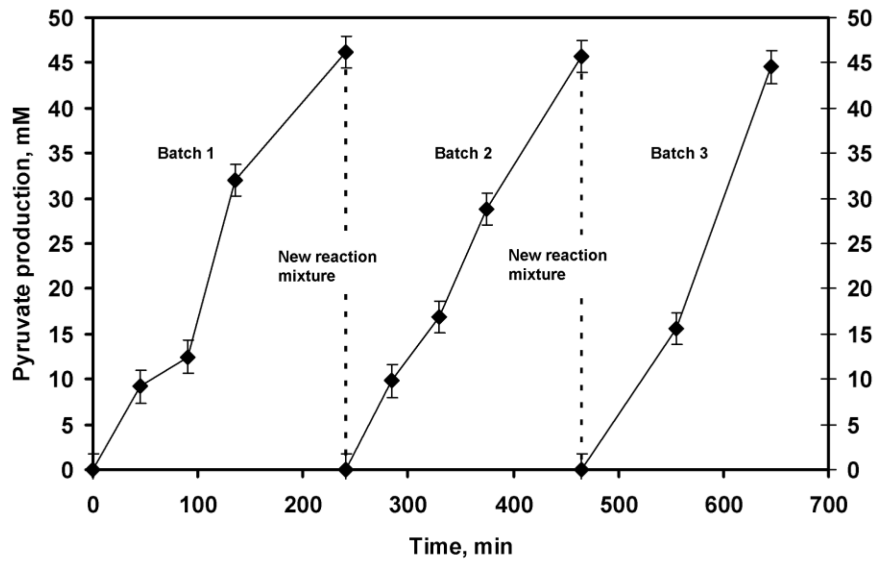


Figure 4



Supporting information

Oriented Enzyme Immobilization On Functionalized Silica Carrier Using the Cationic Binding Module *Z*$_{basic2}$: Design of a Heterogeneous D-Amino Acid Oxidase Catalyst on Porous Glass

Juan M. Bolivar and Bernd Nidetzky

Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

* Corresponding author
E-mail: bernd.nidetzky@tugraz.at; Phone: +43 316 873 8400; Fax: +43 316 873 8434

Running title: *Z*$_{basic}$-mediated immobilization of D-amino acid oxidase

Abbreviations: DAO; D-amino acid oxidase; *Tv*DAO; DAO from *Trigonopsis variabilis*

SUPPORTING MATERIALS AND METHODS

S.1 Carrier Surface Functionalization

Controlled pore glass (CPG) was activated with amino (CPG-amino) (Howarter and Youngblood 2006), epoxy (CPG-epoxy) (Porsch 1993), or sulfonate groups (CPG-sulfonic) (Athens et al. 2009) by using protocol slightly modified from the given literature. Typically, 100 mg of CPG previously washed with 3 N NaOH and distilled water were incubated under mild stirring in 1 mL of a freshly prepared aqueous solution of a silanization reagent that is specified in Table S1 where the reaction conditions are also indicated. The pH was adjusted with concentrated HCl/NaOH. After the reaction, the resulting modified CPG was washed extensively with water.

Table S1

Table 1. Silanization of CPG

Activated CPG	Silane agent	Conditions of reaction
CPG-amino	**10 % (v/v) APTES**	**pH 6.0, 80 ºC, 3 h**
CPG-epoxy	**5 % (v/v) GPMES**	**pH 8.5, 25 ºC, 16 h**
CPG-sulfonate	**10 % (v/v) SPTHS**	**pH 8.0, 80 ºC, 3 h**

3-Aminopropyl-triehoxysilane (APTES), (3-glycidoxypropyl) trimethoxysilane (GPMES); 3-(trihidroxysilyl)-1-propane-sulfonic acid (SPTHS)

Activation of CPG-epoxy with carboxylate groups (CPG-carboxylate)

CPG-carboxylate was prepared by incubating CPG-epoxy with L-aspartic acid. The surface epoxy groups of the carrier react with the amino group of Asp (Batalla et al. 2008). One hundred mg of CPG-epoxy were incubated in 1 mL of 3 M Asp (disodium salt) at pH 8.5 for 16 h at 25 ºC. Finally, the activated carrier was washed with water.

Activation of CPG-amino with carboxylate or sulfonate groups (CPG-C_4-carboxylate, CPG-C_4-sulfonate)

Modification of CPG-amino was performed using glutardialdehyde (Migneault et al. 2004; Walt and Agayn 1994). One hundred mg of CPG-amino was incubated in 1 mL of 10 % (v/v) glutaraldehyde in water, the pH was adjusted to 7.0, and the reaction was left for 3 h at 25 ºC. After this time, the carrier was extensively washed with water and further incubated in 1 mL of 1 M glycine or amino sulfonic acid at pH 8.0 for 3 h, yielding CPG-C_4-carboxylate and CPG-C_4-sulfonate, respectively.

S.2 SDS-PAGE analysis of immobilized *Tv*DAO

Soluble and immobilized preparations of Z_{basic2}_*Tv*DAO were analyzed by SDS-PAGE as previously described (Laemmli 1970). The immobilizates were boiled in Laemmli's disruption buffer that contains mercaptoethanol and SDS, thus releasing from the support any bound protein. Samples were analyzed in 12% polyacrylamide gels and detected by using staining with Coomassie.

Supporting figures.

Figure S1.

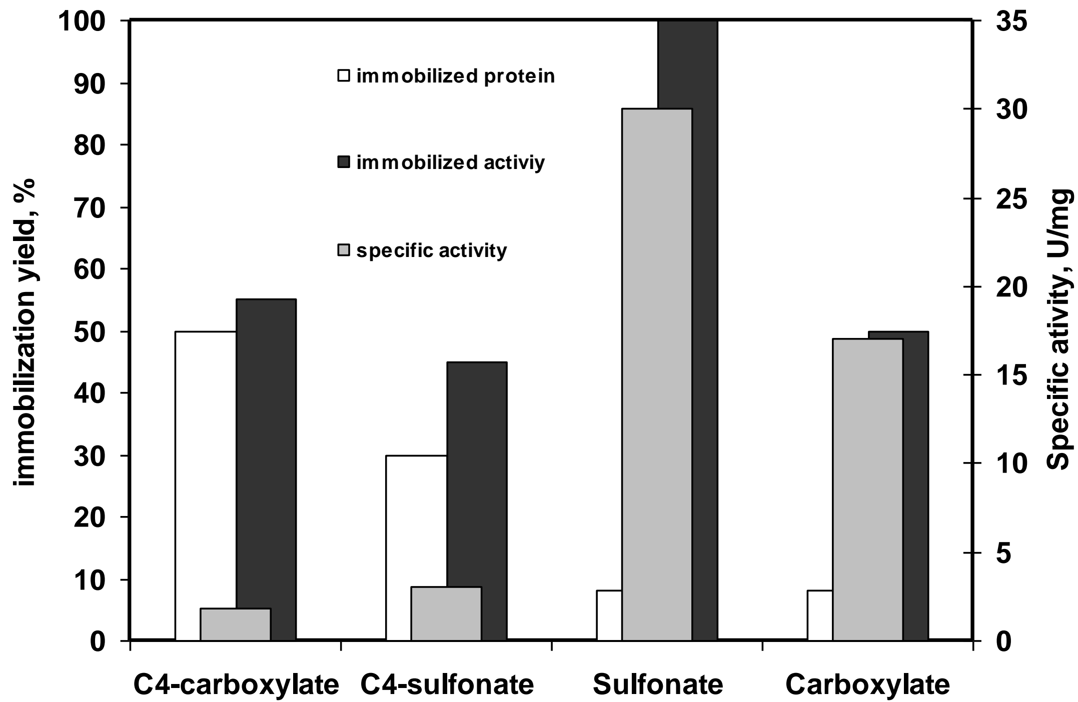


Figure S1. Yield of immobilization of Z_{basic2}_*Tv*DAO on activated CPG, and specific activity of the resulting enzyme immobilizates. The immobilization yield is expressed as protein/activity immobilized divided by offered protein/activity present in crude extract. Experiments were carried out with 0.1 g carrier/mL of suspension in 50 mM potassium phosphate buffer at pH 7.0 at 25 ºC. About 200 units of activity and 100 mg of protein were offered per gram of carrier.

Figure S2

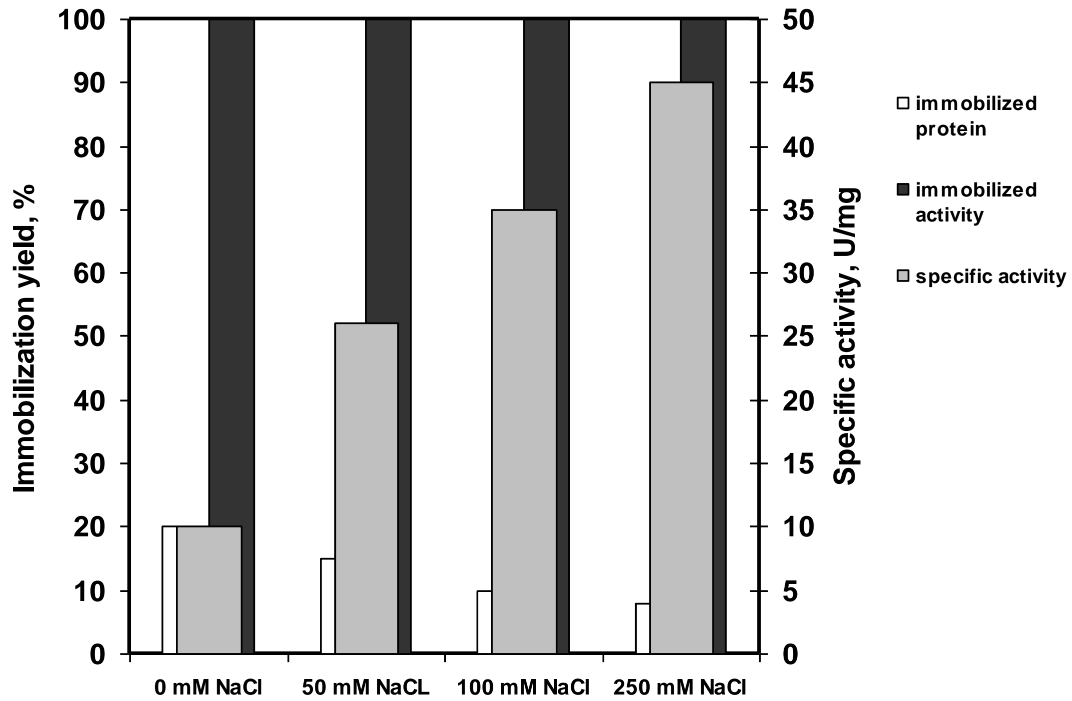


Figure S2. Effect of ionic strength on immobilization yield and specific activity of immobilized Z_{basic2}_*Tv*DAO for immobilization on CPG-sulfonate. Experiments were carried out with 0.1 g carrier/mL suspension in 10 mM potassium phosphate buffer at pH 7.0 at 25 ºC. About 200 units of activity and 100 mg of protein were offered per gram of carrier.

Figure S3

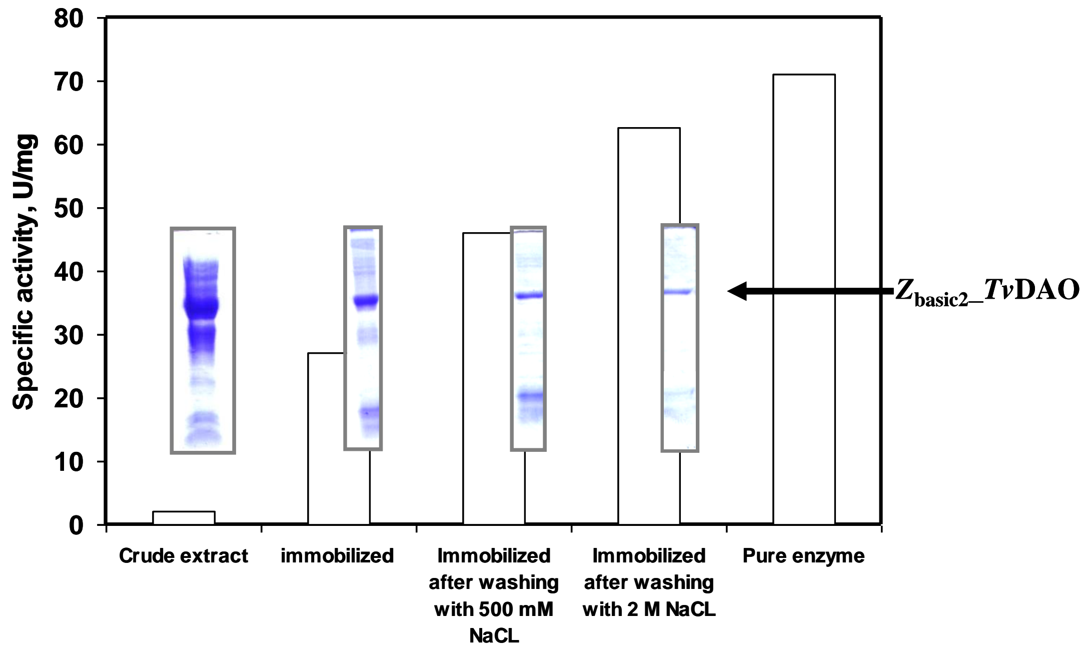


Figure S3. Reversible immobilization of Z_{basic2}_*Tv*DAO on CPG-sulfonate monitored by specific activity measurement and SDS PAGE. Immobilization was performed at pH 7.0 using a 10 mM potassium phosphate that contained 250 mM of NaCl. The resulting immobilizate was washed with the same buffer containing different concentrations of salt and the eluted protein and activity were measured. The specific activity of the immobilized Z_{basic2}_*Tv*DAO was calculated from these measurements, and the data in the figure are complemented by results of analysis of the immobilized enzyme by SDS PAGE.

Figure S4

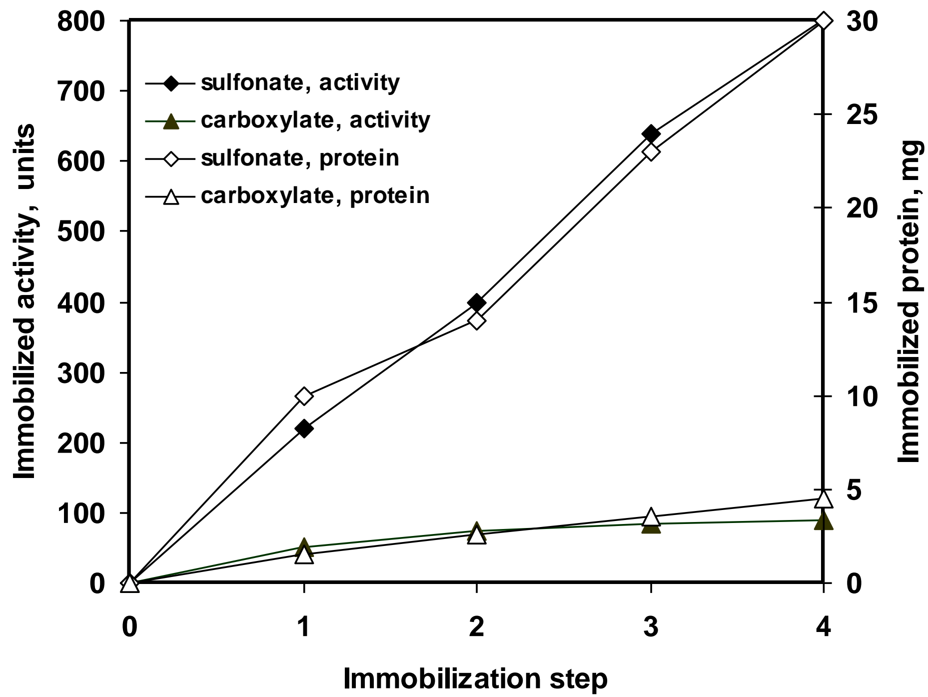


Figure S4. Immobilization of Z_{basic2}_*Tv*DAO on CPG-sulfonate in several loading steps. Experiments were carried out at pH 7.0 and 25 ºC using a 10 mM potassium phosphate buffer that contained 250 mM NaCl. About 110 mg of protein and 220 units of activity were offered per g of carrier and in each round.

Supporting references.

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