The positively charged mini-protein Z_{basic2} as a highly efficient silica binding module: opportunities for enzyme immobilization on unmodified silica supports

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ABSTRACT. Silica is a highly attractive support material for protein immobilization in a wide range of biotechnological and biomedical-analytical applications. Without suitable derivatization, however, silica surface is not generally usable for attachment of proteins. We show here that Z_{basic2} (a three α-helix bundle mini-protein of 7 kDa size that exposes clustered positive charges from multiple arginine residues on one side) functions as highly efficient silica binding module (SBM), allowing chimeras of target protein with SBM to become very tightly attached to underivatized glass at physiological pH conditions. We used two enzymes, D-amino acid oxidase and sucrose phosphorylase, to demonstrate direct immobilization of Z_{basic2} protein from complex biological samples with extremely high selectivity. Immobilized enzymes displayed full biological activity, suggesting that their binding to the glass surface had occurred in a preferred orientation via the SBM. We also show that charge complementarity was the main principle of affinity between SBM and glass surface, and Z_{basic2} proteins were bound in a very strong, yet fully reversible manner, presumably through multipoint non-covalent interactions. Z_{basic2} proteins were immobilized on porous glass in a loading of 30 mg protein/g support or higher, showing that attachment via the SBM combines excellent binding selectivity with a technically useful binding capacity. Therefore, Z_{basic2} and silica constitute a fully orthogonal pair of binding module and insoluble support for oriented protein immobilization, and this opens up new opportunities for the application of silica-based materials in the development of supported heterogeneous biocatalysts.

KEYWORDS. Protein immobilization; silica support; underivatized glass; non-covalent attachment; cationic binding module; charge complementarity; oriented and selective binding

BRIEFS. The positively charged mini-protein Z_{basic2} is an efficient silica binding module that allows protein immobilization on unmodified silica surface through selective non-covalent interactions.

INTRODUCTION

Silica-based materials constitute an extremely versatile class of inorganic supports for the immobilization of proteins in a wide range of biotechnological and analytical applications [1-8]. Their use extends from the structured elements of microarrays and microfluidic devices, to industrial bioreactors. The different applications encompass a variation in operating volume from the low microliter to the multi-cubic meter scale [1, 3, 6]. Therefore, silica supports are fabricated in different degrees of structural precision and functional sophistication. Their costs vary widely in accordance with the commercial production scale, depending on the respective application. Even though the exact chemical composition of the silica material changes in dependence of both the source and the type of processing used, there is the common feature that the surface of the support contains slightly acidic silanol groups (Si-OH; pK_a ≈ 6 – 7) [9-12]. At neutral pH in aqueous solution, therefore, deprotonation of the silanol moieties results in the development of a negatively charged surface, imparting it the character of a weak cation exchanger [10-12]. However, the silica surface is also somewhat hydrophobic due to the siloxanes contained in the structure [11-14]. Therefore, binding of a protein to underivatized silica could involve electrostatic, hydrophobic, hydrogen bonding as well as van der Waals interactions[11-12, 14-15]. It was shown in several studies that these surface properties of silica are not generally suitable for the direct immobilization of proteins in a controlled manner [3-5, 10, 11, 14, 15]. This is a critical limitation for the use of silica supports in their “native” form.

As a solution, different strategies were proposed that are distinguished according to whether the improvement of compatibility between the silica support and the target protein had resulted from modification of the support surface, the protein, or both [3, 4, 16, 17]. To clarify the current need for technology development in the field, it is helpful to summarize the main characteristics of what might be considered an “ideal” method of protein immobilization. [18-21] 1) Attachment of the protein to the support interferes minimally with protein function. 2) A common principle of protein-surface interaction is exploited and therefore, the immobilization is adaptable to many of different proteins using a single protocol. Additionally, the immobilization is also selective and therefore, unprocessed protein samples

such as crude cell extracts can be employed in a combined purification-immobilization approach. 3) No chemical derivatization of the support surface is necessary. 4) Protein binding is tight, preventing leakage from the support under conditions of use; yet, the immobilization is non-covalent and therefore readily reversible under suitable elution conditions. Re-use of the protein, the support or both is therefore possible. 5) The support material is chemically and mechanically robust. Its internal microstructure (e.g. pores in silica particles; micro-channels in structured plates) provides a suitable amount of accessible surface for protein binding.

The vast majority of the reported protocols of protein immobilization on silica support fail in one or more of the key characteristics just described [3, 4, 8, 16, 17, 22-25]. Some few examples of work with underivatized support (e.g. for protein purification)[22, 25-27] notwithstanding, requirement for surface modification probably represents the most commonly encountered limitation. Note that silica surface modification is a well-established technology in principle and the literature offers a large body of materials chemistry and engineering science for this purpose [3, 28-30]. However, fabrication of a silica support harboring a spatially uniform and chemically well-defined functionalization of its surface usually requires a multi-step synthetic procedure that is technically complex and whose practical implementation therefore remains a challenge. Aside from the extra costs generated in the process, a particular restriction of commonly used procedures of surface modification is their limited scalability. For example, silica modification using silane reagents offers the possibility to introduce a variety of surface groups for covalent or non-covalent attachment of proteins. While this method is routinely applied at the laboratory scale, it causes significant problems of reagent handling and reaction control in micro-scale devices and at the industrial scale of process operation. Finally, silica surface chemistry needs to be adjusted to specific protein requirements (e.g. charge, hydrophobicity).

We would like to communicate herein a new approach of protein immobilization on silica that is in excellent accordance with the stated requirements, where in particular it offers the advantage of work with the unmodified silica support. The approach explores the use of a silica-binding module (CBM) that is linked in a single polypetide to the protein of interest, thus targeting it to the silica surface. The concept seems to be quite general in respect to its applicability to different proteins, as we shall

demonstrate with two representative examples selected from enzymes used in industrial biocatalysis. The concept originated from our recent studies on the engineering of enzymes for non-covalent and thus reversible immobilization on anionic supports [31, 32]. We proposed construction of a protein chimera, in which the target enzyme is fused to a strongly positively charged mini-protein called Z_{basic2}. Z_{basic2} 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 [33-35]. The p*I* of Z_{basic2} is calculated from the sequence as 10.5 [34]. Even though this strategy requires a previous and successful work of genetically engineering the target enzyme, the general suitability of the construction of Z_{basic2} fusion protein has been previously evaluated and several examples of fused protein can be found in literature [33-35]. The fusion to Z_{basic2} did not compromise recombinant production of the target proteins in *E. coli*, the activity and stability of the fusion enzymes were also unaltered [31]. We have shown that enzymes harboring Z_{basic2} were attached with high affinity to supports displaying negatively charged sulfonate groups on their surface whereas the corresponding "parent" enzymes were unable to bind to the same supports in significant amounts [31, 32]. It was confirmed that binding of Z_{basic2} enzymes occurred with excellent selectivity and in a highly preferred orientation via the Z_{basic2} module. Immobilized enzyme preparations therefore retained essentially all of the specific activity of the enzyme in solution [31, 32].

During a screening of supports for the immobilization of Z_{basic2} enzymes, unmodified glass did not emerge as candidate support. Even though Z_{basic2} enzymes were attached somewhat efficiently to the anionic glass surface at neutral pH, the selectivity of binding was extremely low (see the Results section below). It also appeared that the binding was due to a variety of interactions, not primarily mediated by Z_{basic2}. However, we were encouraged by a series of recent studies of an alternative silica-binding protein, also termed the Si-tag, in which authors could show dramatic enhancement of the selectivity of target protein binding through variation of immobilization conditions [36-40]. Like Z_{basic2}, the Si-tag is characterized by a large positive net charge (resulting from the relative abundance of basic residues) that appears to be decisive for its binding to silica [36, 39]. However, in marked contrast to Z_{basic2}, which is a relatively small and structurally stable module, the Si-tag is composed of 273 amino acids and belongs to a family of intrinsically disordered proteins that are characterized by an inherently flexible structure

in solution [36, 39]. It was proposed that conformational adaptation of the Si-tag concomitant with its binding to the silica surface could be the key for the observed tight and selective attachment of Si-tag fusion proteins to the solid support [39]. Therefore, even though work with the Si-tag presents conceptual precedence for the use of a positively charged binding module for targeting proteins to silica surfaces, there is a high enough structural and perhaps also mechanistic difference between Z_{basic2} and the Si-tag to clearly warrant this study. This notion is also strongly upheld by results from earlier works in which silica binding of proteins equipped with an oligo-arginine tag was examined [41]. While the oligo-arginine tag did promote the binding, the actual affinity of the tagged proteins to become surface-attached was not very high [36-41]. It seems therefore that interactions between the positively charged groups on the protein and the silica surface need to be optimized for high-affinity binding.

We show in this work that Z_{basic2} has properties of an almost ideal SBM, and the combination of Z_{basic2} and unmodified glass gives a fully orthogonal pair of binding module and insoluble support for protein immobilization in high affinity and selectivity. We discuss structural properties of Z_{basic2} governing its excellent SBM function.

MATERIALS AND METHODS

Materials

TRISOPERL® glass beads, henceforth termed controlled pore glass (CPG), were obtained from VitraBio GmbH (Steinach, Germany) with the following specifications: 50-100 µm diameter, 161.2 nm pore size, 1520.91 mm^3/g pore volume, and 43.16 m^2/g specific surface area. Molecular weight marker proteins for SDS-PAGE were from Amersham Biosciences [GE Healthcare] (Chalfont St. Giles, United Kingdom). All other materials used were of reagent grade.

Enzymes

D-Amino acid oxidase from *Trigonopsis variabilis* (*Tv*DAO), and sucrose phosphorylase from *Leuconostoc mesenteroides* (*Lm*SPase) were used. Enzyme production was done recombinantly in *E. coli* as described in earlier works [31, 32]. Each enzyme was obtained in a chimeric form that had the Z_{basic2} module fused at the N-terminus. Construction, isolation and characterization of the Z_{basic2} forms of

*Tv*DAO and *Lm*SPase were reported in a recent paper from this laboratory [31]. For control, we also obtained *Tv*DAO and *Lm*SPase in a form that did not contain Z_{basic2} and is referred to herein as "native". Note, however, that native constructs contained N-terminal tags for facilitated purification (*Strep*-tag II in *Tv*DAO, Strep_*Tv*DAO; His-tag in *Lm*SPase; His_*Lm*SPase) [31]. It is not critical for this work, but nevertheless supportive to know that fusion to Z_{basic2} did not affect negatively the intrinsic activities of *Tv*DAO and *Lm*SPase in their native form. [31] If not mentioned otherwise, we applied *E. coli* cell extract containing the relevant enzyme. This was obtained by disrupting the cells, suspended in a twofold volume of potassium phosphate buffer (pH 7.0), in a French press. Cell debris was removed at 18407 *g* of centrifugal force for 1 h at 4 °C. The clear supernatant was used. The protein concentration was typically between 10 and 50 mg/mL. Purified enzymes containing or lacking Z_{basic2} were used as reference and prepared by reported protocols. The following analytical enzymes were used: horseradish peroxidase (Sigma-Aldrich, Vienna, Austria); rabbit muscle phosphoglucomutase (Sigma-Aldrich); glucose 6-phosphate dehydrogenase (NAD^+ dependent; from *Leuconostoc mesenteroides*; Sigma-Aldrich).

Immobilization of $Z_{basic\ 2}$ enzymes on CPG

The CPG beads were first washed with NaOH (0.3 M) and exhaustively rinsed with water. They were then incubated under mild stirring in 50 mM of potassium phosphate having the pH of further use. *E. coli* cell extract was brought to pH and supplemented with NaCl and Tween, as indicated under the Results. The CPG (~100 mg) was mixed with 1 mL of the cell extract and incubated under gentle mixing at 25 °C. Samples (50 µL) were taken at certain times, either from the supernatant after sedimentation of the beads or from the entire mixture while the beads were still in suspension. Enzyme activity and protein content of the supernatant were determined in each case. Immobilization yield % is calculated as $100 \times (c_0 - c)/c_0$, where c_0 is the initial protein concentration or the initial enzyme activity in solution; and c is the protein concentration or enzyme activity in solution after the immobilization. The beads were washed with different buffers (1 mL), and protein and activity eluted under these

respective conditions were measured. The protein bound to CPG was analyzed directly using SDS-PAGE under reducing conditions (1 mM β-mercaptoethanol), applying reported methods [31, 32].

Assays

Protein was determined with the Bio-Rad Protein Assay referenced against BSA. The assay was applied to determine protein concentrations in the range 0.0.5 – 0.8 mg/mL. Enzyme activities were obtained from initial rate measurements that recorded O_2-dependent oxidation of D-methionine (*Tv*DAO) and glucosyl transfer from sucrose to phosphate (*Lm*SPase). The assay for oxidase activity was applied to soluble and immobilized preparations of *Tv*DAO. It involved a coupled reaction between *Tv*DAO and peroxidase at 30 °C and pH 8.0, performed in the presence of 4-aminoantipyrine and *N*,*N*-dimethylaniline as described in literature [42]. The absorbance at 565 nm was measured. The assay for soluble transferase activity was performed that reaction of *Lm*SPase at 30 °C and pH 7.0 was coupled to the conversion of the α-D-glucose 1-phosphate product by phosphoglucomutase and glucose 6-phosphate dehydrogenase, resulting in the production of NADH (measured at 340 nm) and 6-phospho-D-gluconic acid. Full details of the assays used are given elsewhere [31, 43]. One unit of activity is therefore defined as the amount of enzyme that produces 1 μmol of H_2O_2 (*Tv*DAO) or glucose 1-phosphate (*Lm*SPase) under the standard conditions applied. The preparations of Z_{basic2}_*Tv*DAO used in this work showed specific activites of about 2 U/mg (cell extract) and 71 U/mg (purified enzyme). For Z_{basic2}_*Lm*SPase, the corresponding specific activites were 17 U/mg and 110 U/mg, respectively.

Catalytic effectiveness of immobilized Z_{basic2}_*Tv*DAO

We define catalytic effectiveness of the immobilized enzyme (η) as ratio of the *observable* activity of the immobilizate ($V_{observable}$; U/$g_{carrier}$) 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 enzyme 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 1.0 – 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 horseradish peroxidase (10 µg/mL) was incubated in the presence of immobilizate at 30 °C and under agitation 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.

Structural analysis of Z_{basic2}

Structure modeling of Z_{basic2} was done using the NMR structure of Z domain of staphylococcal protein A [44] (Protein Data Bank entry 2SPZ) as the template. Model of Z_{basic2} was constructed by sequence alignment of Z domain and Z_{basic2} [34] using Modeller 9v8. Visualization was done using pymol 0.99rc6.

Data Analysis and Presentation

The shown data are from multiple independent experiments, typically three or more. Mean values were calculated from the data and are given together with the corresponding standard deviation in text and figures.

RESULTS AND DISCUSSION

Optimizing binding conditions of Z_{basic2}_*Tv*DAO on CPG in high affinity and selectivity

When *E. coli* cell extract containing Strep_*Tv*DAO was incubated with CPG at pH 7.0 (50 mM of potassium phosphate buffer), all of the offered enzyme activity and about half of the total protein were bound to the support. Therefore, this indicated that protein adsorption on CPG occurred somewhat efficiently, yet in a highly non-selective manner. To examine the chemical nature of the interactions involved in *E. coli* protein binding to CPG, we performed elution experiments under conditions representing a systematic variation in the pH and the ionic strength (resulting from addition of NaCl). As neither pH change to high or low values nor increase in NaCl concentration promoted desorption of

the bound protein (Table S1 in Supporting Information), we concluded that the protein adsorption was not primarily driven by electrostatic interactions. This notion was confirmed by showing that addition of 250 mM NaCl did not change the characteristics of enzyme and protein binding to CPG (Figure 1). The non-ionic detergent Tween 20 was therefore used to examine the possible role of hydrophobic interactions in the adsorption of Strep_*Tv*DAO and the accompanying *E. coli* protein to CPG [5]. Interestingly, Tween 20 was highly effective in releasing bound protein and activity from CPG, as depicted in Table S1. Making use of perturbance ("shielding") [5] of hydrophobic interactions by Tween 20, we were able to establish conditions in which essentially no protein from the *E. coli* cell extract was adsorbed to the glass support (Figure 1). With these conditions, we next investigated the binding of Z_{basic2}_*Tv*DAO.

The surface silanol group in glass is expected from literature to have a pK_a in the range 6 – 7 [5, 10-12]. Optimization of opposite-charge complementarity between the glass surface and the Z_{basic2} binding module therefore involves the pH as a key variable. We examined adsorption of Z_{basic2}_*Tv*DAO at different pH values in the range 6.8 – 8.0. In addition to Tween 20, which was always present, NaCl was optionally included in a concentration of 1.0 M to probe the ionic strength dependence of adsorption of protein and enzyme activity. By doing a time course analysis of the binding, we established that adsorption on CPG had reached its apparent equilibrium after an incubation time of about 1.5 h. Data given in Figure 2 refer to this equilibrium. We observe that both pH and ionic strength are determinants of affinity and selectivity of Z_{basic2}_*Tv*DAO adsorption to CPG. The trend that slightly basic pH conditions favored binding of Z_{basic2}_*Tv*DAO is consistent with the notion that optimum adsorption of the cationic binding module requires the surface silanol groups of the CPG to be largely ionized. The binding of total protein was also enhanced at high pH. However, a close look at the respective pH dependencies revealed that increase in pH affected the binding of enzyme activity differently than it affected the binding of total protein. In particular, while change in pH from a value of 7.5 to 8.0 brought about substantial (twofold) enhancement of protein binding, the amount of adsorbed enzymatic activity was slightly affected by the same pH change. Increase in ionic strength through addition of 1.0 M NaCl

changed the pH dependence of binding of enzyme activity whereas it did not affect the binding of total protein. At pH 8.0 with salt present, therefore, all of the offered enzyme activity was bound to CPG, this reflecting an almost twofold enhancement of activity binding as compared to pH 7.5 (Figure 2). Interestingly, the effect of NaCl on promoting adsorption of the enzyme activity was clearly dependent on pH, being highly significant at pH 8.0 while vanishing completely at pH 6.8. Figure S1 shows that a useful NaCl concentration range is 1.0 – 2.0 M. Collectively, these results emphasize the importance of establishing a suitable window of operation with respect to pH and ionic strength for the immobilization of Z_{basic2}_*Tv*DAO. They also show that the effect of NaCl on increasing the selectivity of binding of Z_{basic2}_*Tv*DAO is entirely due to a coresponding positive effect of the salt on the affinity of binding of the Z_{basic2} enzyme. This stands in sharp contrast to the effect of Tween 20, which functions in the suppression of binding of the accompanying *E. coli* proteins. [5] The role of NaCl in promoting the adsorption of Z_{basic2}_*Tv*DAO is probably complex, conceivably including effects of the salt on the Z_{basic2} structure in solution or when bound to CPG. [5, 45, 46] Ikeda and colleagues reported selective binding of Si-tagged proteins to silica particles using conditions similar to the ones described here for Z_{basic2}_*Tv*DAO [36-39]. However, the presence of NaCl in concentrations of up to 2.0 M was described mainly in regard to avoiding the adsorption of untagged proteins. Considering the findings for Z_{basic2}, it could be interesting to examine the potentially positive effect of added salt on the binding affinity of the Si-tag.

Reversibility of binding of Z_{basic2}_*Tv*DAO on CPG

Binding of Z_{basic2}_*Tv*DAO on CPG is non-covalent, and it should therefore be fully reversible in principle. Even though reversible binding offers the advantage of facilitated recovery of the enzyme activity as well as regeneration of the support for manifold re-use, there is the potential problem of wash-out of the adsorbed enzyme under operational conditions. We therefore examined the stability of adsorption of Z_{basic2}_*Tv*DAO to CPG, having in mind that ideally one could switch from conditions of quasi-permanent retention to others that allow for complete elution. Table 1 shows results of experiments in which recovery of protein and enzyme activity was measured for different elution

conditions, representing variation in pH as well as type and concentration of salt. Increase of the NaCl concentration to 5.0 M resulted in elution of all of the adsorbed enzyme activity at pH 8.0 while slightly more than half of the bound protein was recovered. A similar effect was achieved by decreasing the pH to a value of 7.0, leaving the NaCl concentration at 1.0 M. It was shown by Ikeda et al. that substitution of NaCl by $MgCl_2$ facilitated recovery of Si-tagged enzymes from silica supports. [36-39] The superior performance of $MgCl_2$ was explained by a displacement effect that is exclusive to the divalent cations and results from their adsorption on the support surface. We therefore used 5.0 M $MgCl_2$ at pH 7.0 and obtained a slight enhancement of total protein recovery (Table 1). However, no enzyme activity could be recovered under these conditions, presumably because Z_{basic2}_*Tv*DAO was not stable and eluted in an inactive form. An interesting observation was that irrespective of the pH chosen in the range 6.0 – 8.0, activity was not eluted from the support using NaCl in concentration of up to 5.0 M, *unless* Tween 20 was added to the buffer (Table 1). This finding might imply that the original adsorption of Z_{basic2}_*Tv*DAO on CPG was mediated not only by charged contacts but also involved a substantial amount of hydrophobic interactions [5, 10, 11, 47, 48]. An alternative, favored explanation is that high salt concentration (5.0 M) do effectively hinder the mainly charged interactions between Z_{basic2}_*Tv*DAO and silica support, but at the same time they promote alternative hydrophobic interactions that stabilize the bound state of the protein [45, 46, 48].

We can apply wash conditions that do not cause elution of enzyme activity to remove other protein from the surface and thereby enhance the specific activity of the immobilized enzyme (Table 2). Z_{basic2}_*Tv*DAO recovered from the support after washing was almost pure by criterions of specific activity (65 U/mg) and migration as single protein band in SDS PAGE (Figure S2). In conclusion, therefore, we established simple "wash-on" and "wash-off" protocols for Z_{basic2} enzyme immobilization, characterized by extremely stable and selective attachment of the target enzyme to the support while offering at the same time the possibility of facile re-use of (any) silica material used.

Evidence that Z_{basic2}_*Tv*DAO is bound to CPG in a preferred orientation via the cationic binding module, resulting in a fully active immobilized enzyme

A major potential advantage of achieving control over orientation with which enzyme is attached to insoluble support is that immobilized catalyst preparation largely retains intrinsic functionality of soluble enzyme (e.g. turnover frequency, selectivity, binding recognition). In two recent studies of Z_{basic2} enzyme immobilization on supports *functionalized* with "orthogonal" surface sulfonate groups [31, 32], we presented evidence from effectiveness factor (η) analysis that protein binding had probably occurred exclusively via the cationic binding module, leaving the associated enzyme function completely intact. [20, 21] Because it is impractical to extrapolate from sulfonated surface to underivatized silica surface, we measured η for various CPG immobilizates of Z_{basic2}_*Tv*DAO differing in the amount of enzyme activity loaded per gram of CPG. To this end, enzyme immobilization was performed in a multi-step loading experiment, whereby 130 U/g and 96 mg protein/g were offered in each step. Figure 3A shows that all of the applied enzyme activity was attached to the support. Expressing binding selectivity as the ratio of activity and protein attached to the insoluble support in each immobilization step, we can see from Figure 3A that increased loading of the CPG surface did not cause interference (e.g. due to surface crowding). Even though utilization of CPG to full capacity was not attempted in the experiment, ability to load around 30 mg protein/g support suggests that immobilization of Z_{basic2}_*Tv*DAO on underivatized silica could be practially relevant for use in the biocatalysis field. [19, 20, 49] Note that insufficient binding capacity is a common critical issue in the development of selective immobilization methods. Z_{basic2}_*Tv*DAO immobilizates obtained in the different loading steps (Figure 3A) were used for characterization, and Figure 3B shows dependence of η on amount of immobilized enzyme activity. At very low enzyme loading η approached a value of unity, in accordance with expectation for the ideal case of oriented immobilization. Therefore, even on underivatized silica support, surface binding of Z_{basic2}_*Tv*DAO appears to have been highly directed and primarily mediated by Z_{basic2} module. In Figure 3B, marked drop of η at high (i.e. technologically relevant) enzyme loadings likely reflects progressive control of the observable reaction rate by diffusional restrictions. Limitation especially by the O_2 transport rate is exemplified in several studies of (random) immobilizates of O_2-dependent enzymes. A useful strategy to minimize diffusion effects is design of a suitable support structure where in particular

pore diameter and geometry play a decisive role, as these two parameters control reactant effective diffusivity. We therefore would like to point out important recent progress in the materials chemistry field where various silica supports featuring tailored mesoporous structure have been developed. [15, 17, 49, 50] Because such supports are highly promising for enzyme immobilization, availability of a complementary ("orthogonal") technology of attaching enzymes on the silica surface will be crucial to harness the full potential of these supports in the future. We propose fusion of target enzyme to Z_{basic2} module.

How generally useful is protein immobilization on CPG via the Z_{basic2} module? *Lm*SPase as a second example

To underpin general applicability of the conceptual idea developed by using Z_{basic2}_*Tv*DAO, we selected a second enzyme (*Lm*SPase), fused it to Z_{basic2}, and examined binding of the resulting protein chimera to CPG. It is important to note here that *Lm*SPase is a monomeric protein, unlike *Tv*DAO that functions as a homodimer. The biologically active unit of Z_{basic2}_*Tv*DAO therefore contains two binding modules, allowing for particular multivalency in the interaction with silica surface that will not be available to Z_{basic2}_*Lm*SPase. This is likely the reason that under stringency conditions optimized for immobilization of Z_{basic2}_*Tv*DAO, binding of Z_{basic2}_*Lm*SPase to CPG was less efficient than that of Z_{basic2}_*Tv*DAO (Figure 4). However, by lowering the NaCl concentration to attenuate the charge screening effect, we were able to attach Z_{basic2}_*Lm*SPase in a useful yield, as shown in Figure 4. The specific activity of immobilized Z_{basic2}_*Lm*SPase (~80 U/mg bound protein) was close to the specific activity of the purified soluble enzyme (~110 U/mg), indicating that despite the relatively low salt concentration used, immobilization had been highly selective for the Z_{basic2} protein: just about 10% of the total offered protein was attached to the support.

We incubated CPG immobilizate of Z_{basic2}_*Lm*SPase under different conditions to examine stability of protein-surface interaction. In addition, we wanted to explore the possibility of doing "enzyme purification" in a post-immobilization step, applying intermediate washing to remove other proteins from the surface, as described above for Z_{basic2}_*Tv*DAO. At pH 7.0, Z_{basic2}_*Lm*SPase remained stably

attached to CPG up to NaCl concentrations of about 1.0 M. At higher salt concentrations, however, the enzyme was gradually washed off. Using additionally 0.5% Tween 20 in the buffer, all of the enzyme activity was eluted already at 1.0 M NaCl. Therefore, recovery of bound enzyme and/or silica support is readily accomplished by washing with a suitable combination of NaCl and Tween 20. Intermediate washing using buffer (pH 7.0) that contained NaCl in the range 0.25 – 0.50 M successfully enhanced the specific activity of bound Z_{basic2}_*Lm*SPase up to a value expected for purified soluble enzyme in the fully active form, as shown in Figure S3. As with Z_{basic2}_*Tv*DAO, repeated loading of Z_{basic2}_*Lm*SPase yielded a highly active CPG immobilizate (Figure 4). Interestingly, the number of immobilization steps required to obtain a representative protein loading (e.g. 32 mg/g CPG) was much lower when working with Z_{basic2}_*Lm*SPase (2 steps; Figure 5) as compared of Z_{basic2}_*Tv*DAO (6 steps). This observation can probably be attributed to different expression levels for the two Z_{basic2} enzymes in *E. coli*; the relative abundance of Z_{basic2}_*Lm*SPase in the offered cell extract (~15% of total intracellular protein) was much higher than that of Z_{basic2}_*Tv*DAO (~2% of total intracellular protein). High-yielding recombinant production of Z_{basic2} enzyme would clearly support subsequent one-step purification and immobilization on silica support from crude protein mixture. Aggregate data for immobilization of Z_{basic2}_*Tv*DAO and Z_{basic2}_*Lm*SPase on CPG underscore clear advantages of the immobilization method developed in this work and reveal excellent potential for it to be applied in other combinations of enzymes and silica supports.

Structural characteristics of Z_{basic2} potentially important for binding to silica

We studied Z_{basic2} at the levels of amino acid sequence and three-dimensional structure for the possible occurrence of motifs potentially relevant for binding to silica. Z_{basic2} is structurally organized in a central ellipsoidal core formed by the three helices of the Z domain. [44] This core contains 48 amino acids and its approximate dimensions are 2.6 × 3.1 × 2.3 nm. It has two extensions on each side, of which one starts from the N-terminus (6 amino acids) and the other forms the C-terminal part of the Z_{basic2} module (4 amino acids). Table 2 summarizes overall composition of the different structural regions of Z_{basic2} regarding charged and hydrophobic amino acids. Positive charges are located mainly in helix 1 and

helix 2. In order to infer potential binding sites from charge density, we examined surface charge distribution of Z_{basic2} in more detail. Figure 6A shows views of the Z_{basic2} ellipsoid obtained by rotating the molecule around its length axis. The analysis reveals that clustering of positive surface charges is prominent on only one side of the protein module. This region involves helix 1 and helix 2, and it comprises 25 amino acids of which almost half (12 residues) carry positively charge: Lys-7, Arg-9, Arg-10, Arg-11, Arg-13, Arg-14, Arg-17, Arg-24, Arg-27, Arg-28, Arg-32, and Arg-35. All these residues are (partially) surface-exposed, covering an area of approximately 2.3 × 3.1 nm on the protein. It is highly probable that interaction of Z_{basic2} with negatively charged surface would take place via the shown surface patch of positively charged residues. The analysis also shows that multipoint noncovalent interaction between Z_{basic2} and the support should be possible in that way. The remaining surface area of the Z_{basic2} core is rather hydrophobic with isolated positive and negative charges in between. Assuming that Z_{basic2} becomes attached to silica mainly via its large and relatively flat "surface patch" of positive charges, a surface-binding mode of Z_{basic2} enzyme is conceivable in which fusion partner is hardly affected by (oriented) attachment of the cationic binding module. A *hypothetical, yet plausible* model of Z_{basic2} binding is depicted in Figure 6B. It seems therefore that unlike the structurally flexible Si-tag for which conformational adaptation was proposed as the principle mechanism of achieving (high) binding affinity, Z_{basic2} might not have to rely on similarly large and global structural rearrangement to become tightly attached to silica surface. The probable existence of two fundamentally different molecular mechanisms of protein binding to negatively charged surfaces is intriguing and certainly merits deepened comparative analysis in the future.

CONCLUSIONS

Development of fully orthogonal pairs of SBM and support surface could bring important innovation to the field of protein immobilization. Not only would it introduce the possibility of rational design in the immobilization development that otherwise relies strongly on case-specific empirical data, but it could

therefore also contribute to substantial shortening of the overall development time. Despite common conception that immobilization is a fairly mature technology, adaptation of known protocols to a particular protein is usually laborious and it is often not successful in a straightforward manner. Oriented immobilization via binding module having target protein as fusion partner potentially eliminates requirement for extensive optimization of immobilization conditions and decreases the likelihood of failure. Widely used and commercialized strategies of immobilization based on specific recognition (e.g. His-tag or Strep-tag systems; systems involving chitin/cellulose/maltose binding modules) fail in various respects when benchmarked against the five criterions elaborated in the Introduction. Evidence is presented in this work that identifies almost ideal orthogonality between underivatized CPG and Z_{basic2} for protein immobilization purpose in a potentially wide range of applications. Scope and practical use of the system were demonstrated, and advantages were pointed out that result from highly stable, yet non-covalent and thus reversible attachment of target protein on silica surface. Full retention of biological activity in immobilized Z_{basic2} proteins gives clear indication that attachment on the CPG surface took place in a preferred orientation, via the cluster of positive charges on one side of the SBM (see Figure 6B). This result is consistent with the structural difference of Z_{basic2} domain and other commercialized systems used in protein purification, unlike short tags based systems, Z_{basic2} domain would offer enough size to create steric hindrances between protein and surface to preserve side interactions[21] but without compromising the payload of the target protein on surfaces. In addition, the negative charge features of the surface and the presumably low pI presented by the target enzyme would disfavor substantially any trend to additional interaction. We recognize huge potential for protein immobilization, particularly at industrial scale, by expanding the Z_{basic2} system to silica-based supports other than CPG, which was used here as a representative model. Not only should this readily be possible with only fine-tuning of immobilization conditions, but it would also promote exploitation of currently untapped types of support (e.g. mesoporous silica materials).

ACKNOWLEDGMENT. J.M.B. is a recipient of a fellowship (Ref. EX2009-0053) from the Spanish Ministerio de Educación. B.N. acknowledges his involvement in the Austrian Centre of Industrial Biotechnology (ACIB) at Graz University of Technology.

SUPPORTING INFORMATION AVAILABLE. Supporting information contains Table S1, Figures S1 - S3. This material is available free of charge via the Internet at http://pubs.acs.org.

FIGURE CAPTIONS

Figure 1. Tween 20 minimizes unspecific adsorption of Strep_*Tv*DAO on glass. Bound activity is shown in dark bars. Bound protein is shown in white bars. For experimental conditions, see the Materials and methods section.

Figure 2. Optimizing the solution conditions for immobilization of Z_{basic2}_*Tv*DAO in high yield and selectivity. Experiments were performed as described under Materials and methods, offering 180 U activity and 96 mg protein per g support in each round of immobilization. A 50 mM potassium phosphate buffer was used.

Figure 3. Evaluation of Z_{basic2}_*Tv*DAO immobilization on CPG in terms of surface loading (A) and catalytic effectiveness of immobilized enzyme (B). In panel A, white points show bound protein and dark points show bound activity.

Immobilization was done using 50 mM potassium phosphate buffer, pH 8.0, containing 1.0 M NaCl. In each round, 130 U and 96 mg protein were applied per g support.

Figure 4. Optimizing ionic strength for Z_{basic2}_*Lm*SPase immobilization in high yield and selectivity. About 4000 U and 230 mg protein were offered per g support.

Figure 5. Immobilization of Z_{basic2}_*Lm*SPase on CPG in repeated loading steps. White points show bound protein. Dark points show bound activity.

Figure 6. Analysis of surface charge distribution in Z_{basic2} module (A), and hypothetical interaction mode of Z_{basic2} with silica surface (B). Panel A depicts orthogonal views of a three-dimensional representation of Z_{basic2} module, according to dihedral third projection. Panel B is a cartoon showing plausible orientation of Z_{basic2} while becoming attached to a flat silica surface. The color coding

is red for positively charged residues, blue for negatively charged residues, and gray for the hydrophobic residues considered in Table 2.

TABLES

Table 1. Protein and activity eluted from CPG immobilizate of Z_{basic2}_*Tv*DAO.

Conditions of elution (pH / NaCl concentration, M)	**Eluted protein, %**	**Eluted activity, %**
6.0 / 5.0	4 ± 2	5 ± 2
7.0 / 1.0	45 ± 5	2 ± 2
7.0 / 5.0	4 ± 2	5 ± 2
7.0 / 0.25; + 0.5 % Tween 20	20 ± 2	0
7.0 / 0.50; + 0.5 % Tween 20	40 ± 5	0
7.0 / 1.0; + 0.5 % Tween 20	50 ± 5	98 ± 5
7.0 / 5.0 M $MgCl_2$ (replacing NaCl)	80 ± 7	n.d.
8.0 / 5.0	4 ± 2	5 ± 2
8.0 / 5.0; + 0.5 % Tween 20	60 ± 6	100

The immobilizate contained 180 U of activity and 8 mg protein bound per g CPG. Immobilization was performed as described under Materials and methods. Elution experiments were done by incubating 0.1 g immobilizate in 1 mL of 50 mM potassium phosphate at the different conditions given in the Table. Incubation time was 1 h.

Table 2. Summary of features of Z_{basic2} primary structure, mapped onto fold elements.

	Amino acids	Positively charged residues (Arg and Lys)	Negatively charged residues (Asp and Glu)	Hydrophobic residues (Phe, Leu, and Pro)	Net charge, pH 7.0
Z_{basic2} module	58	16	8	11	8 (0.137)
N-terminal region	6	1	1	1	0
Helix 1	12	7	2	0	5 (0.583)
Interhelix (1 – 2) region	5	0	0	3	0
Helix 2	13	5	2	2	3 (0.231)
Interhelix (2 – 3) region	4	0	1	1	-1 (0.25)
Helix 3	14	2	2	3	0
C-terminal region	4	1	0	1	1(0.25)

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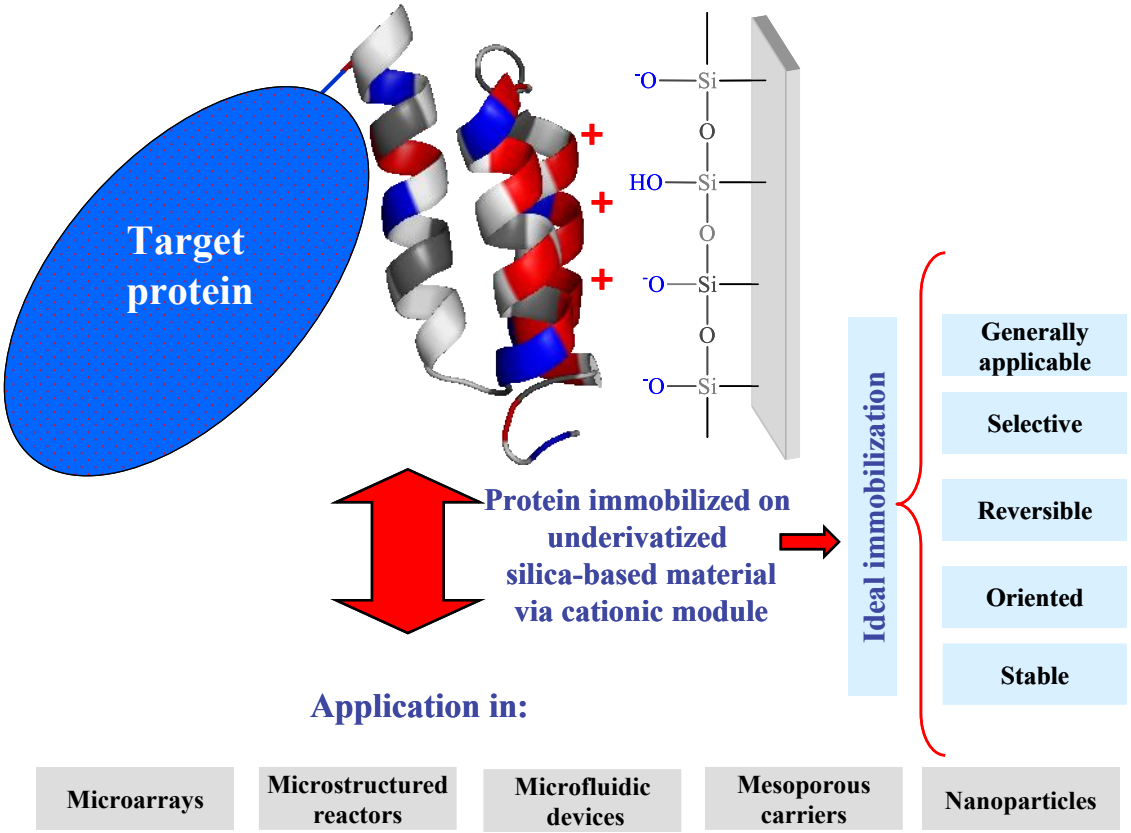
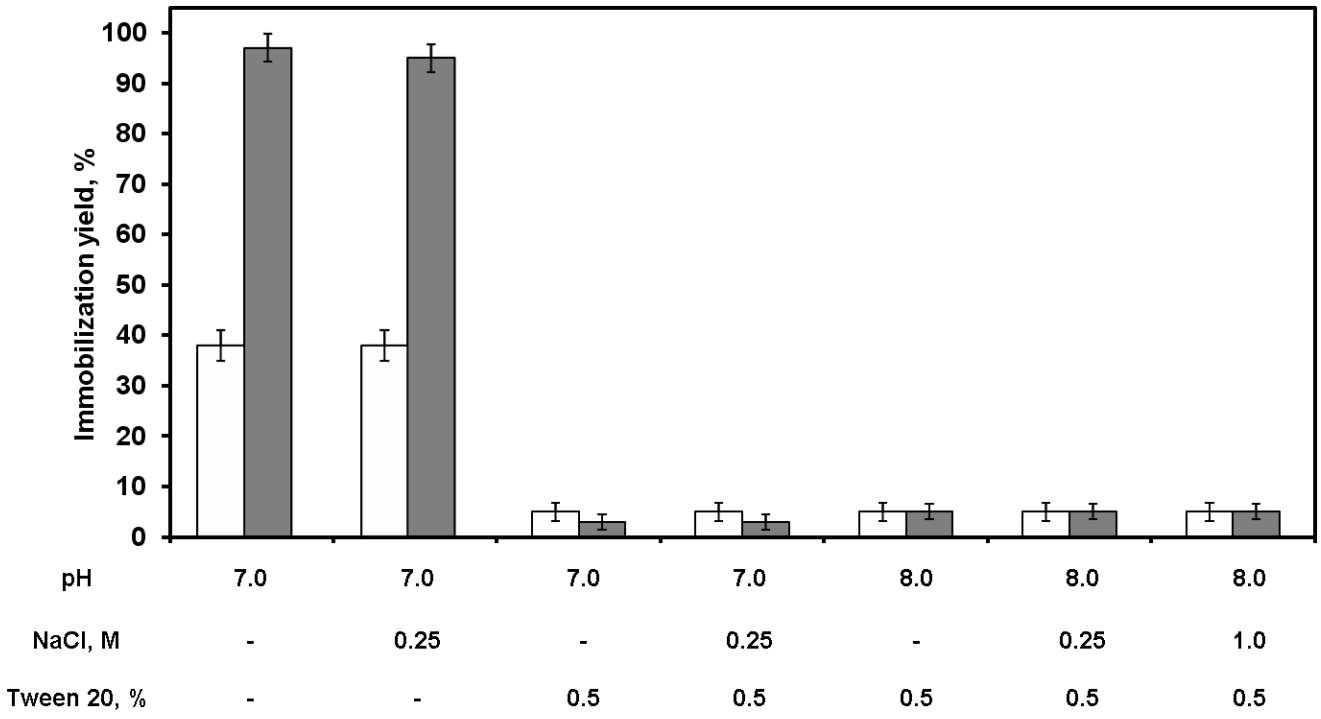


Figures.



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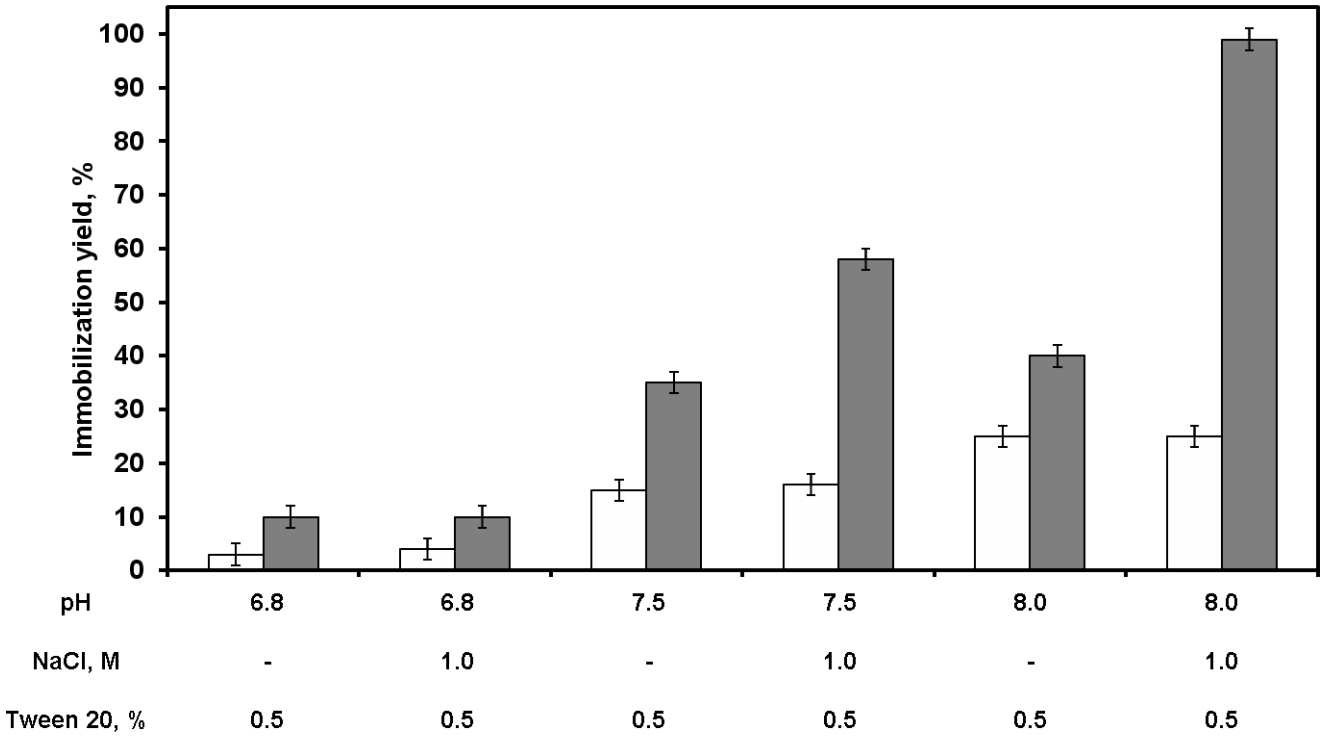
Immobilization yield, %
100
90
80
70
60
50
40
30
20
10
0
pH
6.8
6.8
7.5
7.5
8.0
8.0
NaCl, M
-
1.0
-
1.0
-
1.0
Tween 20, %
0.5
0.5
0.5
0.5
0.5
0.5

Figure 2.

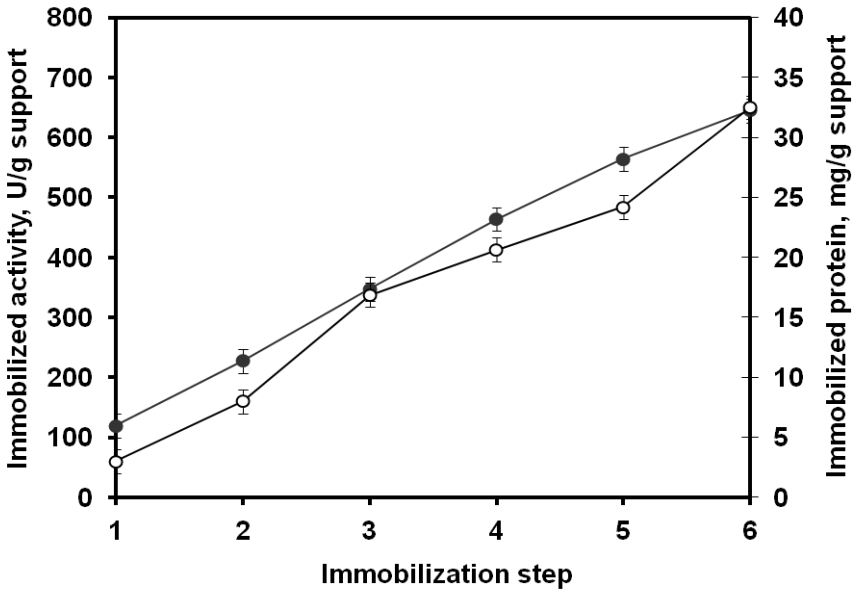

Figure 3 A.

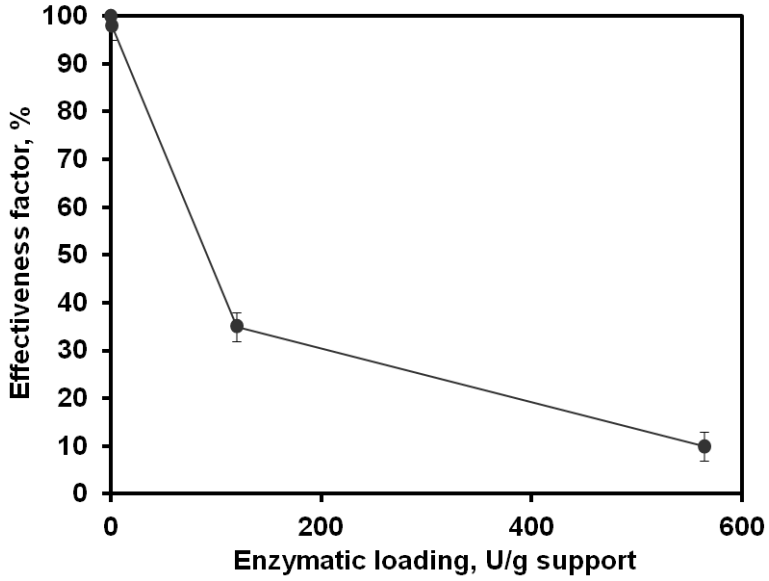

Figure 3 B

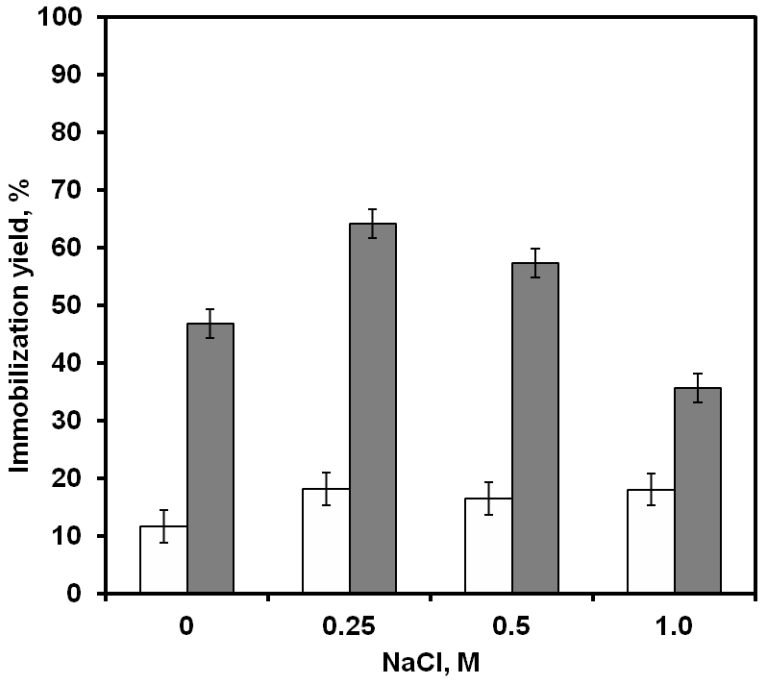
100
90
80
70
60
50
40
30
20
10
0
Immobilization yield, %
0
0.25
0.5
1.0
NaCl, M

Figure 4.

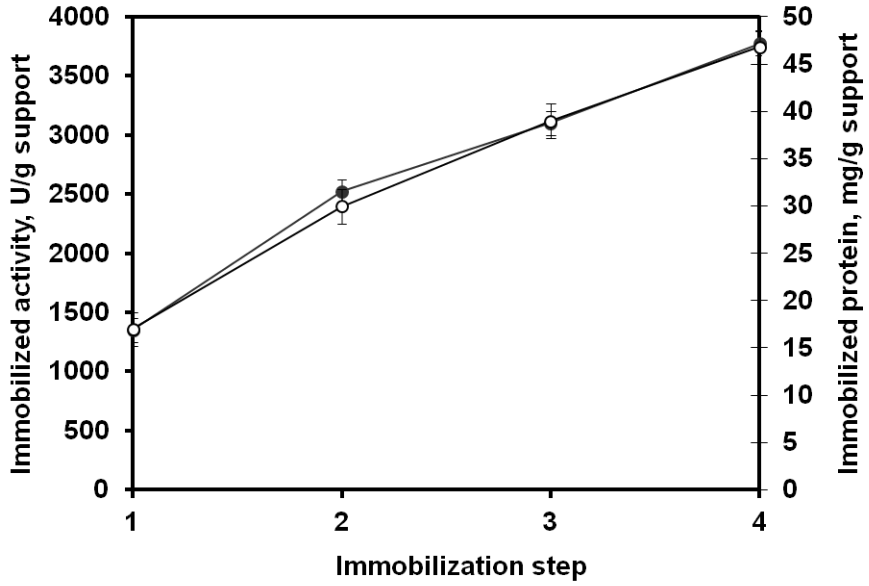
Immobilized activity, U/g support
Immobilized protein, mg/g support
Immobilization step
4000
3500
3000
2500
2000
1500
1000
500
0
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4

Figure 5.

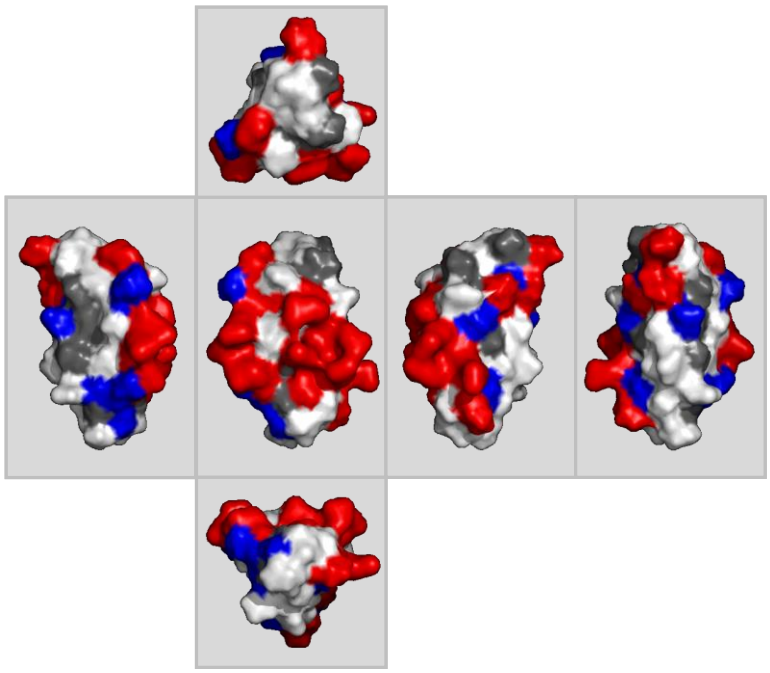

Figure 6A.

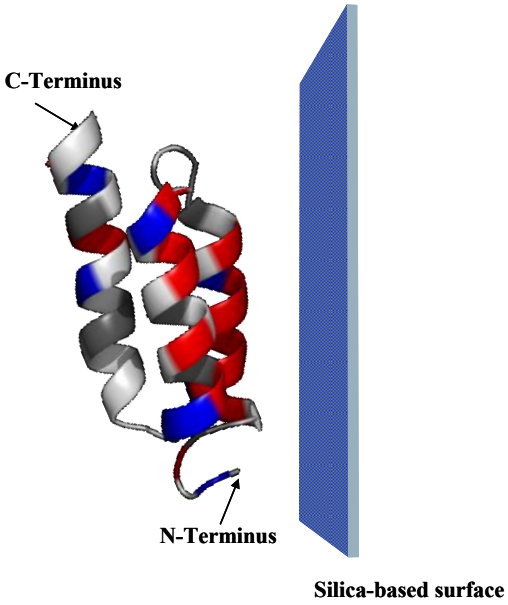

Figure 6B

The positively charged mini-protein Z_{basic2} as a highly efficient silica binding module: opportunities for enzyme immobilization on unmodified silica supports

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TITLE RUNNING HEAD: Enzyme immobilization on unmodified silica supports

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Supporting tables.

Table S1.**Elution of Strep_*Tv*DAO immobilized on glass.**

Conditions of elution (pH / NaCl concentration, M)	**Eluted protein , %**	**Eluted activity, %**
5.5/5	0	0
9.0/5.0	0	0
7.0/5.0	0	0
9.0/5.0 M $MgCl_2$ (replacing NaCl)	0	0
7/0.5 % (w/v) Tween 20	80 ± 7	80 ± 6

The immobilizate contained 150 U activity and 21 mg protein bound per g CPG. Elution experiments were done by incubating 0.1 g immobilizate in 1 mL of 50 mM potassium phosphate at the different conditions given in the Table. Incubation time was 1 h. Eluted protein and activity are expressed are the percentage of protein/activity eluted to the supernatant divided by the protein/activity loaded on glass.

Supporting figures.

Figure S1

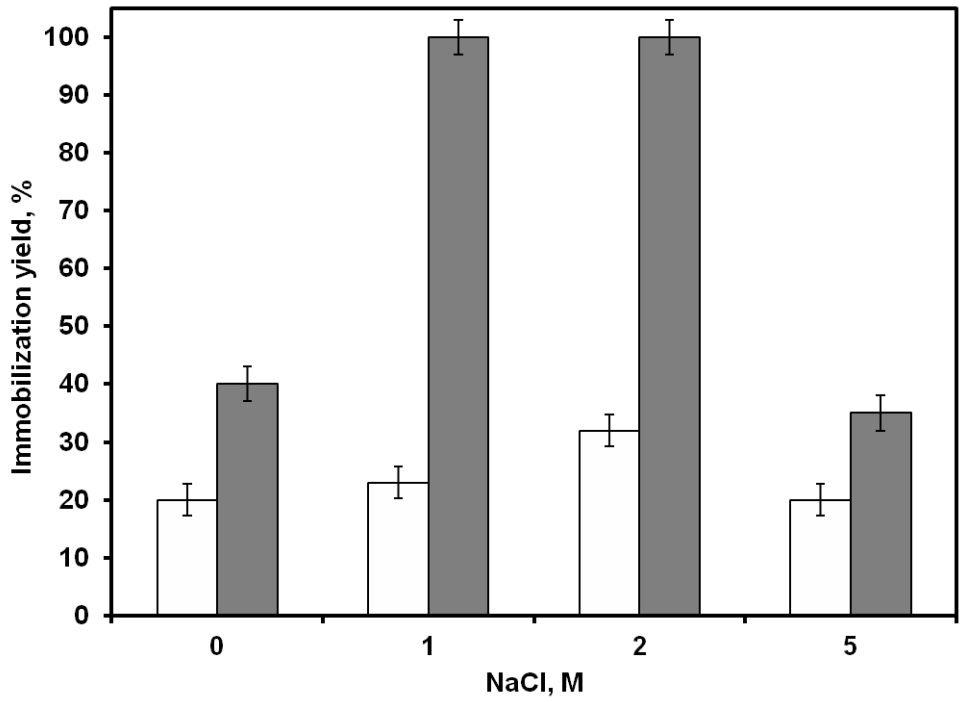

Figure S1. Influence of ionic strength on the immobilization yield of Z_{basic2}_*Tv*DAO. The immobilization yield is expressed as protein/activity immobilized divided by offered protein/activity present in cell extract. Bound activity is shown in dark bars. Bound protein is shown in white bars. Experiments were carried out as described under Materials and methods, using 50 mM potassium phosphate (pH 8.0) and offering 150 U and 80 mg protein per g carrier.

Figure S2.

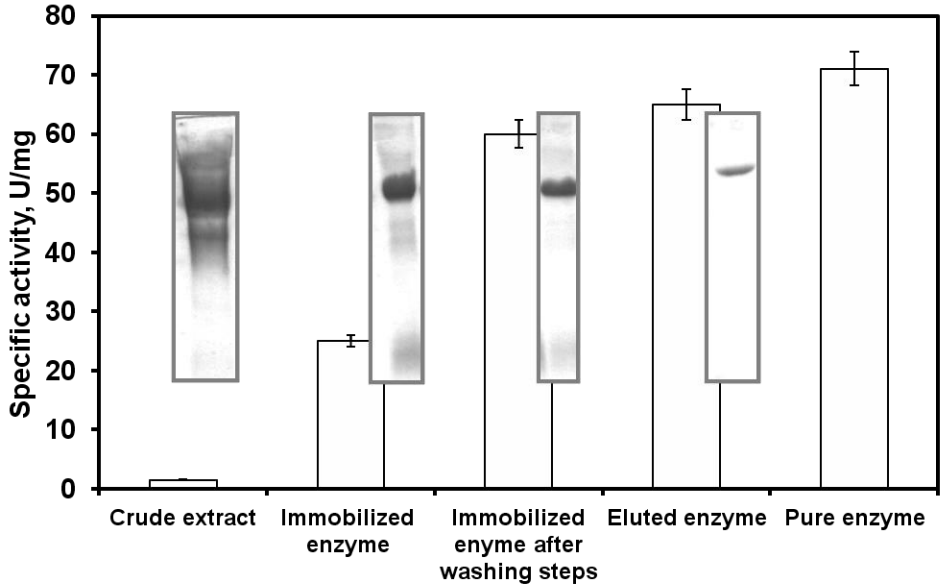

Figure S2. Selectivity of binding of Z_{basic2}_*Tv*DAO on CPG. Immobilization was performed at pH 8.0 using a 50 mM potassium phosphate that contained 1.0 M of NaCl. The resulting immobilizate was washed with the same buffer containing different concentrations of salt and Tween 20, and the eluted protein and activity were measured. The specific activity of the immobilized Z_{basic2}_*Tv*DAO was calculated from these measurements. Data in the figure are illustrated by results of analysis of the immobilized enzyme by SDS PAGE.

Figure S3.

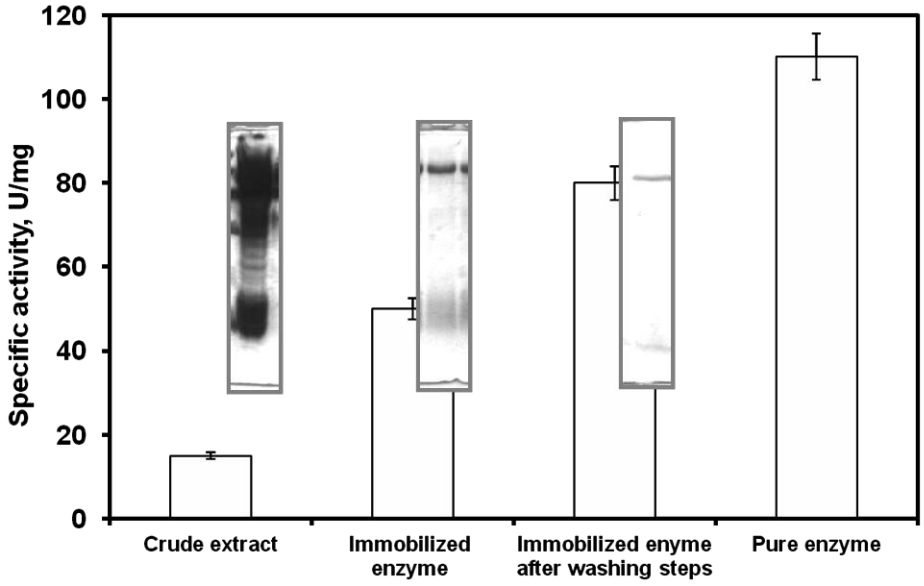

Figure S3. Selectivity of binding of Z_{basic2}_*Lm*SPase onto glass Immobilization was performed at pH 8.0 using a 50 mM potassium phosphate that contained 250 mM of NaCl. The resulting immobilizate was washed with the same buffer containing different concentrations of salt and Tween 20, and the eluted protein and activity were measured. The specific activity of the immobilized Z_{basic2}_*LmSPase* was calculated from these measurements. Data in the figure are illustrated by results of analysis of the immobilized enzyme by SDS PAGE.