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Immobilization of Mercuric Reductase from a *Pseudomonas putida* Strain on Different Activated Carriers

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ABSTRACT

Mercuric reductase was isolated from *Pseudomonas putida* KT2442::mer-73 and immobilized on chromatographic carriers activated by various methods. The immobilization methods for covalent coupling were compared with regard to preservation of enzymatic activity and coupling yields. Highest yields were obtained with carriers bearing the most reactive functional groups. Best results were achieved with tresyl chloride-activated carriers. The optimum binding conditions were found at pH 8.

Application of the immobilized mercuric reductase for continuous treatment of Hg(II)-containing water was examined in a fixed bed reactor. Space-time yields up to 510 nmol/min·mL were attained. The kinetics of immobilized enzyme systems were not diffusion-controlled.

Index Entries: Mercuric reductase; immobilization; eupergit C; cyanogen bromide-; tresyl chloride-; CDI-; oxirane-activated Sepharose 4B.

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INTRODUCTION

Besides natural emissions of mercury by volcanic eruptions and weathering, 15,000 t mercury are presumed to pollute the atmosphere each year by industry leading to contaminations of water and atmosphere with this toxic metal (*1*). Because of its high toxicity it presents a hazard for the environment. The limits for drinking water containing dissolved mercury are fixed by WHO guidelines to 1 μg/L (*1*)

The biological basis for mercury toxicity arises from the binding of Hg(II) to sulfhydryl groups and disulfide bondings, causing inactivation of biologically active molecules, such as enzymes.

The total amount of mercury is released into the environment by anthropogenic or geogenous sources. Therefore, remediation of mercury pollution can be aimed either by altering its ionic form to a less toxic species and/or sequestering it, ideally in forms that can be recycled for further use. Current technologies utilize adsorptive matrices to remove mercuric compounds from industrial effluent. An alternative strategy currently being examined involves the reduction of Hg(II) to the elemental form Hg(0). To this end bacterial mercury resistance is employed that relies on this transformation through the activity of a cytosolic mercuric reductase. Such bacterial reduction systems can eventually be utilized for either on-site remedial situations, since volatilized Hg(0) is both less toxic and less bioavailable at low concentrations, or within contained bioreactors, from which the reduced Hg(0) could potentially be recovered.

Mercuric reductase is an enzyme found in many bacterial strains, e.g., *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas species.* It consists of 3 identical subunits with an approx mol wt of 63,000 Da. It has been found to contain FAD, utilizing NADPH as an electron donor, and requiring an excess of exogenous thiols for activity (*6,10*).

Immobilization of mercury resistant microorganisms was investigated in previous studies (*2*). Difficulties inherent with microorganisms, such as clogging of the fixed bed system with excess biomass, led to problems with the immobilized system in continuous mode. Instead of making use of immobilized microorganisms an alternate approach could be to directly employ the enzyme in immobilized form.

Therefore, the object of this study was the covalent immobilization of mercuric reductase onto different types of activated carriers. Various immobilization methods were investigated in order to compare immobilization levels and maintenance of enzymatic activity. As the enzyme is O_2-sensitive, immobilization methods utilizing functional groups with different reactivity were chosen as compiled in Table 1. The enzyme was isolated from *Pseudomonas putida* KT2442::mer-73. This strain hyperexpresses constitutively merTPAB genes (*3*).

The immobilized enzyme was utilized in a fixed bed reactor in order to evaluate retention of catalytic properties of immobilized mercuric reductase in a continuous process.

Table 1
Immobilization Methods

Method	Reactivity of activated support	Immobilized functional groups at protein surface	Stability of linkage
Sepharose 4B			
Epibromohydrine	moderate	NH, NH_2, SH	good
Bisoxirane	moderate	NH, NH_2, SH	good
1, 1'-Carbonyldiimidazole	moderate	NH, NH_2	unstable at pH > 10
Tresyl chloride	high	NH, NH_2, OH, SH	good
Cyanogen bromide	high	NH, NH_2, OH, SH	stable at 5 < pH < 10
Eupergit C	moderate	NH, NH_2, SH	good

MATERIALS AND METHODS

Materials

Mercuric reductase was isolated from *Pseudomonas putida* KT2442::mer-73 (*4*). The following chemicals were purchased from Fluka: EDTA, Sepharose 4B, 2,2,2-trifluoroethane-sulfonyl chloride (tresyl chloride), 1,1'-carbonyldiimidazole (CDI), epibromohydrine and 1,4 butanedioldiglycidylether (bisoxirane, 70%). Reagents for microbicinchoninic acid (BCA) protein assay were purchased from Pierce. Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia, Freiburg, Germany, Eupergit C from Röhm Pharma, Darmstadt, Germany, Perloza MT 200 was a kind gift of Severoske Chemiche Zavody, CSFR. NADPH and β-mercaptoethanol were obtained from Sigma, Deisenhofen, Germany. All other chemicals were purchased from E. Merck, Darmstadt, Germany. Analytical grade was used in all cases.

Preparation of Cell-Free Extract

Cells were resuspended in 50 m*M* potassium phosphate buffer (50% w/v), pH 7.2, 100 μ*M* $HgCl_2$ and 0.5 m*M* EDTA and disrupted with glass beads in a Retch-mill for 45 min at 4°C. The suspension was centrifuged at 27000*g* for 30 min at 4°C in order to remove solid cell fragments. Enzyme containing supernatant was stabilized by adding glycerol (15% v/v).

Purification of Mercuric Reductase

Cell-free extract was treated at 60°C for 10 min. Precipitated proteins were removed by centrifugation at 27000*g* for 30 min at 4°C. The supernatant was used for immobilization. On average, specific activities between 165 U/g and 200 U/g were detected.

CDI Activation of Sepharose 4B

The activation procedure was carried out according to Bethell et al. (*4*). Briefly, 10 mL Sepharose 4B, after exchanging water with acetone,

were activated with 400 mg CDI in 10 mL anhydrous acetone. The suspension was shaken at room temperature for 20 min and then washed with acetone, acetone/water and finally water.

Epibromohydrine Activation of Sepharose 4B

Sepharose 4B (8 mL) were shaken with 3.25 mL 2*M* NaOH, 5 mL distilled water, and 1.4 g epibromohydrine for 3 h at 30°C and washed with distilled water. The reaction was performed according to E. Hochuli et al. (*5*).

Bisoxirane Activation of Sepharose 4B

Sepharose 4B (10 mL) were added to 5 mL 1*M* NaOH containing 10 mg $NaBH_4$. Finally, 2 mL bisoxirane (70%) were added. The reaction proceeded at 30°C for 8 h with gentle agitation. After activation the carrier was washed with distilled water (*6*).

Tresyl Activation of Sepharose 4B and Perloza MT 200

Activation with tresyl chloride was performed as described by Nilsson et al. (*7*). Sepharose 4B (5 mL) or Perloza MT 200 were activated by 150 μL 2,2,2 trifluorethane-sulfonylchloride.

Coupling of Mercuric Reductase on Activated Carriers

The activated Sepharose 4B carriers were washed with coupling buffer. Two milliliters of suction-dried Sepharose was incubated with 2 mL heat-treated enzyme solution in 50 m*M* potassium phosphate buffer at different pH containing 100 μ*M* $HgCl_2$, 0.05 m*M* EDTA and 15% glycerol (v/v).

Coupling onto CDI-activated Sepharose 4B was done at 4°C for 44 h at pH 7.2.

Coupling onto epibromohydrine-activated Sepharose 4B was carried out at room temperature, pH 10, for 16 h.

Coupling of bisoxirane-activated Sepharose 4B was done at room temperature, pH 8.5, for 18 h.

Tresyl and Cyanogen bromide-activated Sepharose 4B were incubated for 17 h at 4°C and pH 8.

For application in a fixed bed reactor 2 mL of tresyl-activated Sepharose 4B or the same amount of tresyl-activated Perloza MT 200 were incubated with 8 mL heat-treated enzyme solution for 21 h at 4°C and pH 8. The immobilization of the mercuric reductase to tresyl-activated Perloza MT 200 was accomplished according to the Sepharose 4B protocol.

Coupling of Mercuric Reductase on Eupergit C

Two milliliter mercuric reductase solution was applied to 0.2 g dry carrier. Immobilization was achieved at pH 7.2 for 16 h and room temperature.

Treatment of Carriers After Coupling

Each carrier was washed with 0.5*M* NaCl after binding in order to remove nonspecifically adsorbed protein. Then the support was treated with 1% β-mercaptoethanol in 0.1*M* Tris HCl, pH 8, in order to inactivate remaining functional groups.

Enzyme Assays

The activity of both soluble and immobilized reductase preparations was determined as described by Fox and Walsh (*8*). Assays were carried out at 37°C in 25 m*M* potassium phosphate, pH 7.2, 133 μ*M* NADPH, 100 μ*M* $HgCl_2$, and 1 m*M* β-mercaptoethanol. The reaction was started with NADPH. The oxidation of NADPH was followed spectrophotometrically at 340 nm at 37°C. Reduction of 1 μmol Hg(II) causes oxidation of 1 μmol NADPH.

Kinetic parameters were determined through variation of $HgCl_2$ concentration.

Protein Assay

Specific activity of Hg-reductase was calculated from total activity and protein mass as determined by the BCA assay from Pierce.

Variation of Amount of Enzyme and Purification Grade

The interference of other proteins on the immobilization yield of reductase on tresyl-activated Sepharose 4B was judged by immobilization of cell-free extract and heat-treated cell-free extract, respectively. Furthermore, various amounts of enzyme solution were used for the covalent immobilization on tresyl-activated Sepharose 4B.

Storage Stability

Immobilized reductase was stored at 4°C in (a) 50 m*M* potassium phosphate buffer pH 7.2, containing 0.5 m*M* EDTA, and (b) additionally 1% β-mercaptoethanol. The activity was measured after various time intervals.

Application in a Fixed Bed Reactor

A column (Pharmacia, 5 mm diameter) was packed with mercuric reductase immobilized on tresyl-activated Sepharose 4B to a final bed-volume of 0.25 mL.

For the application of higher flowrates 1.0 mL of immobilized tresyl-activated Perloza MT 200 was packed into the same columns as described above.

Various $HgCl_2$-concentrations (90–450 μM) in 50 mM potassium phosphate, pH 7.2, containing 1.0 mM β-mercaptoethanol and 0.2 mM NADPH were pumped through the column reactor at 20°C at different flowrates (0.08 cm/s–0.24 cm/s).

The reaction rate, R, of Hg(II) was calculated according to

$$R = (c_{in} - c_{out}) \cdot u \quad (1)$$

where c_{in} is the inlet concentration and c_{out} the outlet concentration in nmol/mL; u is the flowrate in mL/min. Space-time yields S were received by relating reaction rate and reactor volume V_{Reac} as

$$S = R/V_{Reac} \quad (2)$$

Operational Stability

The operational stability of mercuric reductase on Perloza MT 200 was investigated in the fixed bed reactor over a period of 3 h in 270 μM $HgCl_2$, 45 mM potassium phosphate, pH 7.2, 1 mM β-mercaptoethanol, and 160 μM NADPH at a flowrate of 0.16 cm/s (2 mL/min).

RESULTS AND DISCUSSION

Comparison of Activated Carriers

Table 1 represents a compilation of the different activation methods employed in this study. The functional groups of the reagents are either leaving groups, as for tresyl-activated carriers, or are integrated in the covalent linkage formed after immobilization of the enzyme, as for cyanogen bromide- and epoxy-activated carriers. CDI is also leaving groups; however, a carbonyl group is left at the support forming a carbamate linkage with amino-groups. The reagents vary in their reactivity for functional groups at the protein surface; for example, OH-groups react only with highly reactive groups of cyanogen bromide- and tresyl-activated carriers.

The different activation methods displayed major differences in their reactivity for binding mercuric reductase, as demonstrated in Fig. 1. Comparing with other activation methods, tresyl-activated Sepharose 4B

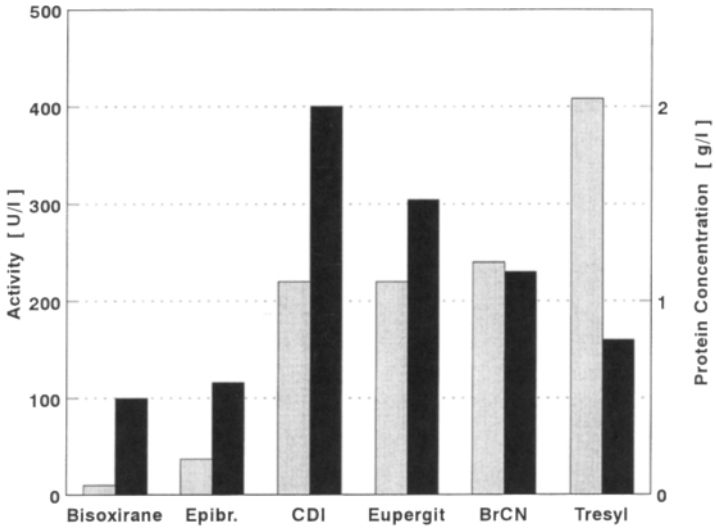

Fig. 1. Total activity of mercuric reductase (left bar) and protein concentration (right bar) immobilized on different activated carriers.

reached both highest immobilization level of enzyme activity and highest specific activity of the immobilized enzyme.

The Sepharose 4B carriers with less reactive oxirane groups, as obtained through activation with epibromohydrine and bisoxirane, demonstrated considerably lower reactivity than cyanogen bromide- and tresyl-activated Sepharose 4B.

Oxirane-activated Sepharose 4B reacts very slowly with proteins at neutral pH. Particularly at a pH close to neutrality, mainly sulfhydryl groups react with oxirane groups. Only at higher pH do amines compete with sulfhydryl groups owing to their higher pk_s-value (*9*). Although the experiments were done in the presence of Hg(II), in order to protect the sulfhydryl groups in the active site, they were obviously linked to the support by this way. This was indicated by scarcely detected enzymatic activity in the supernatant after immobilization. It is very unlikely that all 3 subunits of mercuric reductase are inactivated during immobilization. Hence, one would assume that at least 2 out of 3 should be active. However, the low conversion rates indicate that all 3 subunits are inactive. Considering the mechanism of Hg(II)-transformation, this can be explained. In previous studies it was found that a specific arrangement of the active sites takes place during catalysis forming an intersubunit active site that allows the binding and transformation of Hg(II) (*10*). Such a dependency of different subunits was observed by other investigators, too (*11*).

Eupergit C, although containing oxirane groups as well, reached a much higher immobilization level in relation to both epibromohydrine and bisoxirane-activated Sepharose 4B. Probably, this is because of the higher density of oxirane groups on the surface of the Eupergit carrier (Eupergit C: $\approx$150 μmol/mL, Sepharose 4B: $\approx$20 μmol/mL) providing

higher immobilization levels and less discrimination between functional groups at the protein surface. Therefore, it is more likely that amino groups at the surface of the enzyme reacted, whereas the probability of binding sulfhydryl groups at the active site decreased.

The specific activity of immobilized mercuric reductase at both the cyanogen bromide- and tresyl-activated Sepharose 4B was higher than the dissolved enzyme in heat-treated cell-free extract containing many other proteins. This implies a preferred binding of mercuric reductase in contrast to contaminating proteins. Selective binding was highest with tresyl-activated Sepharose 4B. Therefore this immobilization method was used for further investigation.

Optimum of pH

The optimum immobilization level of the different activation methods displayed distinct dependencies on pH, as demonstrated in Fig. 2. The results are interpreted on the basis of different reactivities of functional groups at the activated carriers and the reactivity of functional groups at the surface of the enzyme, respectively.

Enzymatic activity of immobilized enzymes based on epibromohydrine-activated Sepharose 4B increased slightly with increasing pH, whereas activity based on CDI-activated Sepharose 4B was highest at pH 7.2. The increase in enzymatic activity on the oxirane-based support is owing to a higher amount of uncharged amino groups at elevated pH, leading to a better competition of amino against sulfhydryl groups. Therefore more sulfhydryl groups are left at the active site of the enzyme at higher pH compared to lower pH. However, this increase is rather small because of the instability of the enzyme at elevated pH.

The decline of activity at higher pH on CDI-activated supports is explained by deactivation of CDI groups over reaction time (48 h reaction time). This is in accordance with the literature, where highest immobilization levels on CDI-activated supports were found between pH 4–5 by Wilchek et al. (*12*) and Crowly et al. (*13*).

The optimum pH for immobilization of mercuric reductase on tresyl-activated Sepharose 4B was established at pH 8, corresponding to the highest specific activity. This is attributed to a larger amount of uncharged amino groups at the proteins surface providing more nucleophilic groups for binding. Moreover, very little deactivation of the enzyme can be expected at pH 8 as judged from stability experiments of the free enzyme.

Variation of Enzyme Amount and Purification Grade

The immobilization of heat-treated enzyme led to higher total activities and specific activities in comparison to the nontreated cell-free extract. During heat treatment large amounts of contaminating proteins precipitate, whereas the mercuric reductase concentration decreases only

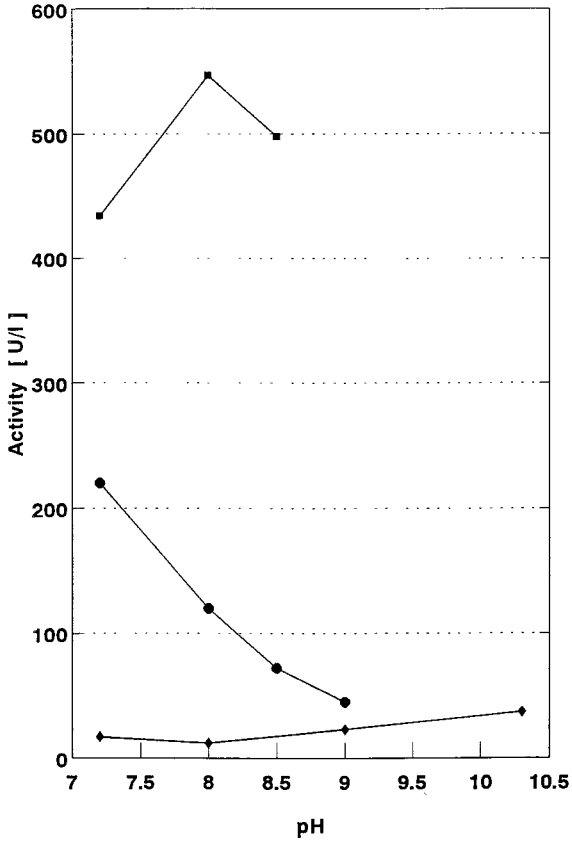

Fig. 2. Total activity of tresyl (■), CDI- (●), and epibromohydrine (◆) activated Sepharose 4B as function of coupling pH. In all cases 2 mL enzyme solution were mixed with 2 mL activated Sepharose 4B. However, initial activities of cell-free extracts differed for each activation method. For tresyl, epibromohydrine-, and CDI-activated carriers 762 U/L (106 U/g), 552 U/L (141 U/g), and 690 U/L (230 U/g) were measured, respectively.

slightly. Therefore less proteins compete for binding sites at the activated carrier leading to considerably higher immobilization levels of the mercuric reductase. The maximum loading of mercuric reductase was reached by application of approx 3 times the volume of the heat-treated enzyme in relation to the volume of the Sepharose gel. The dependence of enzyme amount on activity yield is presented in Fig. 3.

Kinetic Parameters

The results of enzyme kinetics were evaluated graphically from a Michaelis-Menten plot (Fig. 4). $k_{0.5}$ of the free enzyme was determined as 34 μM for $HgCl_2$ (Table 2). k_m for mercuric reductase from other microorganisms is reported between 12 μM (*P. aeroginosa*) and 30 μM (*A. hydrophila*) for $HgCl_2$ by other groups (*6,14*).

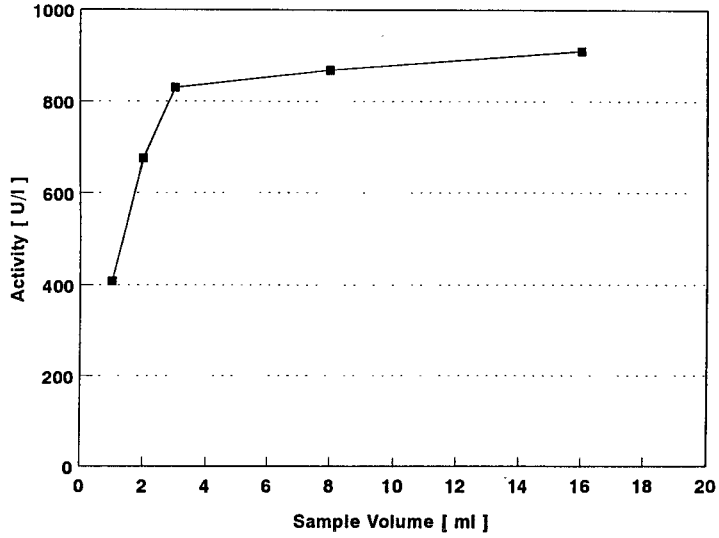

Fig. 3. Influence of sample volume of heat-treated mercuric reductase solution (537 U/L, 188 U/g) on total activity yield of tresyl-activated Sepharose 4B. The reaction was carried out over 17 h at pH 8.0 and 4°C.

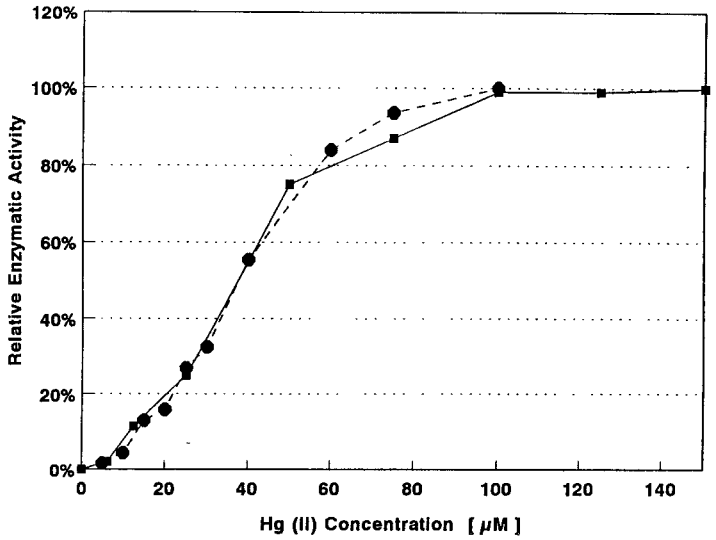

Fig. 4. Kinetics of cell-free extract (●) and immobilized mercuric reductase (■). The reaction was carried out at 37°C in 25 m*M* potassium phosphate buffer, pH 7.2, including 1 m*M* β-mercaptoethanol and 135 µ*M* NADPH. Activities at highest substrate concentrations were set 100%. $k_{0,5}$ of free enzyme: 34 µ*M*, $k_{0,5}$ of immobilized enzyme: 37 µ*M*. V_{max} of free enzyme: 170 U/g, V_{max} of immobilized enzyme: 250 U/g.

Table 2
Kinetic Parameters

	$k_{0,5}$ μM	V_{max}, U/L	Reaction rate,[a] nmol/min 20°C	37°C
Heat treated extract	34[a]	762–879[c]	—	—
Batch mode	37[a]	986[a]	—	—
Fixed bed mode	—	798[b]	38	153

[a]Determined at 37°C.
[b]Determined at 20°C.
[c]Measured with 450 μM $HgCl_2$, at 0.08 cm/min.

Enymes often exhibit changes of k_m after immobilization. Both higher (*15,16*) and lower affinities for the substrate (*17*) are observed. Effects leading to a change of kinetic parameters of an enzyme during immobilization are classified as: conformational and steric effects, partitioning effects, microenvironmental, and mass-transfer effects (*18*). Apparently, none of these effects took place after immobilization of mercuric reductase onto tresyl-activated Sepharose 4B, indicated by very close $k_{0.5}$ values of both the free and immobilized enzyme (Table 2).

The enzyme is concentrated on the carrier surface during immobilization. Furthermore, preference for mercuric reductase was found on tresyl-activated carriers during immobilization, as described above. Hence, V_{max} of immobilized mercuric reductase is slightly higher than with the free enzyme. V_{max} of batch and fixed bed mode cannot be compared directly, since results were obtained at different temperatures. However, a fourfold increase of reduction rates were observed between 20–37°C, anticipating a much higher V_{max} in the fixed bed mode at 37°C than in the batch mode, as expected.

Storage Stability

The stability of the immobilized enzyme on tresyl-activated Sepharose 4B is comparable to the soluble enzyme (half-life: 3 wk). The largest decline of enzymatic activity of immobilized reductase was observed at the beginning of the test when stored in phosphate buffer containing β-mercaptoethanol. After 3 wk almost no further decrease was evident.

Storing the immobilized enzyme in buffer without β-mercaptoethanol resulted in a higher loss of enzymatic activity at the beginning. However, after 14 d the same activity was reached as measured with the β-mercaptoethanol-containing buffer. In contrast, the free enzyme looses its activity immediately in the absence of β-mercaptoethanol or Hg(II) in the storage buffer.

Uo et al. (*19*) investigated the immobilization of mercuric reductase onto various activated porous glass carriers. They also found that stability of the enzyme depends on the immobilization method. Highest stability was found using aryl amino-activated porous glass, whereas the carboxylic derivative lost activity over a period of 10 d. However, also the aryl amino derivate displayed highest decline of enzymatic activity during the first 10 d, which is consistent with the results reported in this paper.

Application in a Fixed Bed Reactor

Elemental mercury was retained in the column causing a grey precipitate to appear at the upper part of the column during operation. The precipitate extended through the column over time without causing an incline of column back pressure. It can be assumed that at least parts of the transformed mercury can be disposed in concentrated form with the chromatographic support.

Experimental results proved that Sepharose 4B was not compressed at flowrates up to 0.08 cm/s (1 mL/min) using the described column system. At $HgCl_2$-concentrations up to 100 μM and a minimum column bed height of 1.2 cm, complete reduction of Hg(II) took place at the maximum flowrate (0.08 cm/s). Therefore calculation of the reaction rate was impossible. To this end, Perloza MT 200 was employed. The cellulose-based support displayed remarkably higher rigidity than Sepharose 4B.

The immobilization level onto both Sepharose 4B and Perloza MT 200 was investigated in batch tests before packing the columns. Perloza MT 200 yielded a lower total activity of the carrier and a lower specific activity as well compared to Sepharose 4B. Besides the carrier structure, which is agarose for Sepharose 4B and cellulose for Perloza MT 200, the main reason should be because of the smaller pore size of Perloza MT 200 in contrast to Sepharose 4B. The approximate fractionation range for Perloza MT 200 is 10 kDa to 1 MDa, whereas for Sepharose 4B 60 kDa to 20 MDa is specified. Since mercuric reductase represents a relatively large protein with a mol wt of 195 kDa, diffusion into small pores is hindered leading to a lower immobilization level and a heterogeneous distribution of the enzyme in the pores of the chromatographic matrix. Owing to the same reason small proteins were preferably immobilized. This explains the lower specific activity of Perloza MT 200 compared to Sepharose 4B. Furthermore, Sepharose 4B exists in smaller particles (40–160 μm) than Perloza MT 200 (110–250 μm), leading to a lower Thiele module and a higher effectiveness factor (*20*). Both supports are known to be biocompatible to a large extent. Therefore microenvironmental effects are not expected.

Kinetics of Hg(II) reduction in the fixed bed reactor depends on flowrate, temperature, column height, and substrate concentrations for a given system. The space-time yields of the enzyme immobilized on Perloza MT 200 are shown in Fig. 5. Space-time yields increased with increasing Hg(II) concentration until inhibition takes place at very high concentrations

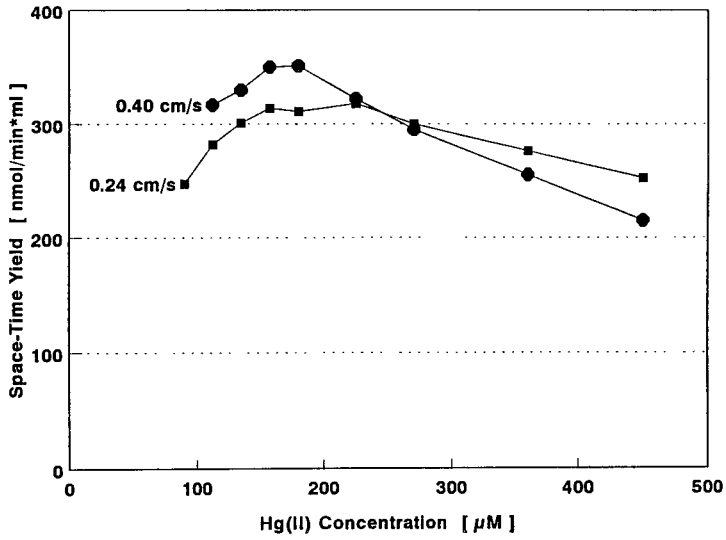

Fig. 5. Influence of flowrate (0.24 cm/s (■) and 0.4 cm/s (●)) on space-time yields of mercuric reductase coupled onto tresyl activated Perloza MT 200 at different substrate concentrations in a fixed bed reactor at 20°C in 50 m*M* potassium phosphate, pH 7.2, including 1 m*M* β-mercaptoethanol and 160 μ*M* NADPH.

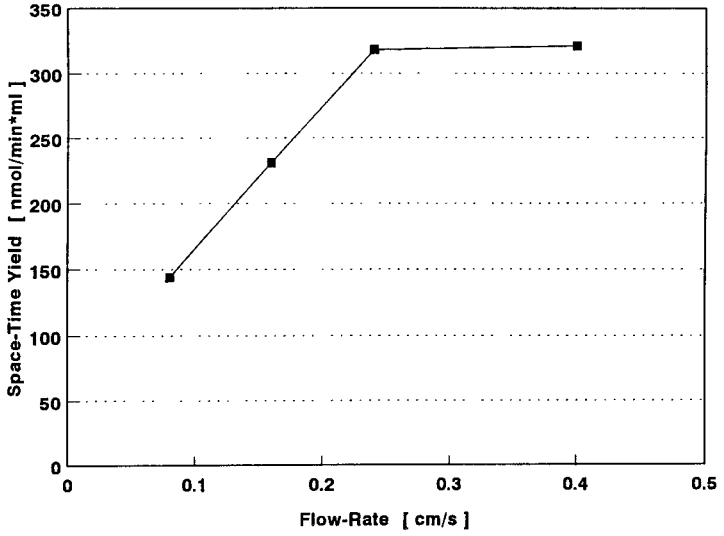

Fig. 6. Influence of flowrate on space-time yield of mercuric reductase coupled onto tresyl-activated Perloza MT 200 at 20°C in 25 m*M* potassium phosphate, pH 7.2, containing 160 μ*M* NADPH, 225 μ*M* $HgCl_2$, and 1 m*M* β-mercaptoethanol.

of mercury (*11*). Substrate inhibition is more dominant at higher flowrates, since the concentration gradient of Hg(II) is lower between column inlet and outlet.

The influence of flowrate on this matrix is displayed in Fig. 6. The increase of space-time yield is proportional to flowrate in the range of 0.08

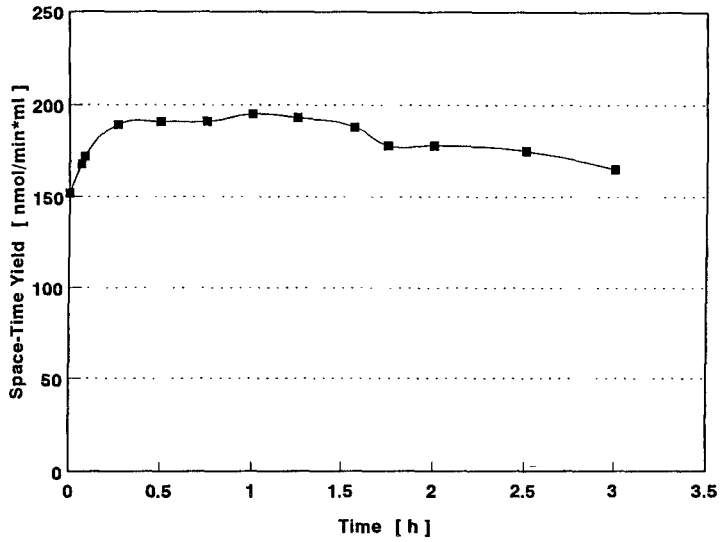

Fig. 7. Operational stability of mercuric reductase coupled onto Perloza MT 200 at 20°C, 270 μM $HgCl_2$, and 160 μM NADPH at a flowrate of 0.16 cm/s.

cm/s to 0.24 cm/s. At higher flowrates saturation with the substrate takes place. Consequently, higher flowrates led to higher outlet concentrations of Hg(II).

At 20°C and 180 μM Hg(II) space-time yields of 350 nmol/min·mL were reached at 0.40 cm/s and 510 nmol/min·mL at 0.08 cm/s with immobilized reductase on tresyl-activated Perloza MT 200 and Sepharose 4B, respectively.

Operational Stability

The operational stability of the fixed bed system was investigated over a period of 3 h only owing to high costs of NADPH. At the beginning of the test the space-time yield increased about 30%, whereupon no change was observed during the following hour. After 1.5 h a decline up to 20% was noticed (Fig. 7). Because of the short time-scale of the experiment it cannot be concluded whether the decrease of enzymatic activity after 1.5 h is attributed through inhibition of the enzyme caused by the formation of Hg(0) or whether it displays a long time oscillation of the packed-bed enzyme system.

SUMMARY

Activation methods on the basis of oxirane groups cannot be recommended since they bind preferably to the SH-groups of the active site of mercuric reductase. Therefore, it is essential to apply highly reactive acti-

vation reagents for immobilization of mercuric reductase, such as cyanogen bromide or tresyl chloride.

Highest immobilization levels of mercuric reductase were achieved by application of tresyl-activated Sepharose 4B and Perloza MT 200 carriers. Space-time yields up to 510 nmol/min·mL and 350 nmol/min·mL were achieved in a fixed bed reactor using Sepharose 4B and Perloza MT 200 carriers, respectively.

The fixed bed mode allows the application of higher Hg(II) concentrations than the free enzyme. Apparently, inhibition by the substrate does not play such an important role compared to free mercuric reductase.

A major drawback of the application of the enzyme is the sensitivity of mercuric reductase against dissolved oxygen. This is critical for waste water treatment. Moreover, the addition of both exogenous thiols and the coenzyme NADPH is inadequate and expensive in a continuous process. Regeneration of NADPH by enzymes or the application of macromolecular NADPH may help to circumvent this problem. Both prospects have been demonstrated and are still in progress (*21,22*). The application of the immobilized mercuric reductase in an enzyme-membrane-reactor would allow regeneration of the cofactor at the same time.

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