Engineering of Efficient Biocatalysts Using Nanostructured Mesoporous Silicate Carriers

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ABSTRACT

Comparative studies of the biocatalysts with invertase immobilized on three nanostructured silicate carriers: mesoporous cellular foams and two types of SBA-15 materials using three highly recommended protein bonding methods are reported. Invertase was attached to the surface by: physical adsorption followed by its crosslinking, adsorption on aminated MS followed by its crosslinking, and covalent bonding with aminated MS via GLA spacer. Experiments clearly indicate that activity of the aminated MCF-based biocatalysts is significantly higher than of both SBA-15-based counterparts. The microenvironment of proteins and porous texture of supports appear the most important factors for enzyme activity expression, and in this respect the unique properties of aminated MCFs can hardly be matched.

Keywords: mesoporous silica, enzyme immobilization, invertase

1 INTRODUCTION

The widespread interest in enzyme immobilization stems from the important practical benefits, e.g. the improved stability of biocatalysts, possibility of their reuse and facile separation from reaction mixtures. The enzymes are usually attached to solid carriers by: covalent bonding, physical adsorption or physical entrapment [1]. Of the three methods the covalent attachment is perhaps the most attractive because the enzymes do not leak out from the support, yet the harsh conditions of bonding may adversely affect the catalysts’ activity. Polymeric organic materials are the most common carriers but owing to disposal problems, poor solvent stability and often poor reusability alternative supports are highly sought [2-4]. More recent studies indicate that the nanostructured mesoporous silicates (MS) are the superior enzyme carriers [5-8]. It is not yet clear which family of MS offers the largest potentials – MCFs, as advised in [5,6], or SBA-15 as recommended in [7,8], and how to attach the enzymes. Herewith, we report the extensive comparative studies of the biocatalysts with invertase (β-fructofuranosidase, EC3.2.1.26) immobilized on: (1) mesoporous cellular foams (MCF), with ultra large, cage-like mesopores and very open structure and (2) two types of SBA-15 materials that feature rod-like mesopores, one with smaller pores of sizes: 7 nm (SBA-15S), recommended as superior enzyme carrier in [7], and another one with larger pores of 22 nm (SBA-15L), advised in [8].

Invertase was bonded with MS by three methods: (1) physical adsorption with the subsequent crosslinking of proteins, (2) adsorption on aminated MS with subsequent crosslinking of enzymes (as recommended in [7]), and (3) covalent bonding using aminated MS and then activated with GLA (recommended in [5,6]). The measurements included: determination of activity of the free enzyme and the biocatalysts prepared in the conversion of sucrose to glucose and fructose, determination of protein loading and the stability of catalysts. The applied test reaction is of practical importance. The sugar mixtures obtained are widely used in the production of noncrystallizing creams, jams, beverages and artificial honey [11].

2 EXPERIMENTAL SECTION

2.1 Materials

Tetraethoxysilane (98%, TEOS), Pluronic P123 and 1,3,5-trimethylbenzene (TMB) used in synthesis of MCFs were from Aldrich so as 2-aminoethyl-3-aminopropyltrimethoxysilane (AEAPTS) applied as donors of amino groups. Invertase in powder (EC 3.2.1.26, Batch IV031037) were kindly donated by Novozymes. Trihydroxymethylaminomethan (Tris), glutaraldehyde (GLA), divinyl, NH4F and other chemicals were purchased from POCh (Poland), and Glukoza OXY DST from Alpha Diagnostics.

2.2 Synthesis and Characterisation of MS

The values of specific surface area, $S_{BET}$; pore volume, $V_p$; diameter of cells, $d_p$ and that of interconnected windows, $d_w$ of pristine (calcined) and modified materials are given in Table 1. They were obtained from nitrogen adsorption isotherms at 77 K (Micromeritics ASAP 2000) using BJH algorithm.
2.2.1 Synthesis of Mesoporous Silica (MS)

Preparation of pristine MCFs was done similarly as described in detail [5], the large pores SBA-15 were made as in [8] and the small pores SBA-15 as described in [9].

2.2.2 Modification of Carrier Surface

Before grafting MCFs were contacted with water vapor for 5 h and subsequently dried at 200°C for 2h. The silanols concentration on the MCFs surface, determined as proposed in [10], was ca. 2.9 OH/nm². Amino groups were grafted onto MCFs surface by reacting the suitable amounts of organosilanes (AEAPTS dissolved in toluene) under reflux (24h, 40°C) with silanols present on the silica surface to obtain the load of functional moiety of about 1.5 mmol/g of silica. In particular, 50mL of the solution containing AEAPTS were stirred under reflux with 1 g of MCF for 24h, after which the solvent was evaporated at 60°C.

2.3 Covalent Immobilization of Invertase

Functionalised carrier (3–5 cm³) was rinsed by centrifugation (6500 rpm, 15 min) five times with distilled water and the 0.1M phosphate buffer pH 7. Then their activation was done in 0.1 M phosphate buffer pH 7 with 2.5 vol.% glutaraldehyde. After activation the carrier was washed, filtered off and it was suspended in 10 mL of proteins in the 0.1 M phosphate buffer pH 6. The slurry was mixed for 2h and stored overnight at 4°C. The excess protein was washed off with a proper buffers: 0.1 M phosphate buffer pH 7; 0.1 M phosphate buffer pH 7 with 0.5 M NaCl, 0.1 M acetate buffer pH 4.5 and finally with a distilled water. All the eluates /supernatants were collected and analyzed for the presence of protein and activity. The amount of bound protein (activity) was calculated from a difference between the amount used for immobilization and that washed off. In order to block unreacted active groups, the filtered preparation was suspended in 0.5 M Tris–HCl buffer, pH 7.8, and then rinsed with 0.1 M acetate buffer prior to activity measurements.

2.4 Physical Adsorption of Invertase with Subsequent Crosslinking

Functionalised and pristine carriers (3–5 cm³) were rinsed by centrifugation (6500 rpm, 15 min) five times with distilled water and the 0.1M phosphate buffer pH 6. After this the carriers were filtered off and suspended in 10 mL of proteins in the 0.1 M phosphate buffer pH 6. The slurry was mixed for 2h and stored overnight at 4°C. The excess protein was washed off with a 0.1 M phosphate buffer pH 7 and then incubated in the same buffer containing 0.25 vol.% glutaraldehyde (GA). After GA treatment for 30 min, the samples were excessively washed by water. The capping of unreacted aldehyde groups was completed in a 0.5 M Tris-HCl buffer, pH 7.8. After Tris-capping, the samples were washed four times by a water and next by 0.1 M acetate buffer pH 4.5 and stored at 4°C.

2.5 Activity Assays

2.5.1 Activity of Native Invertase

Enzyme activity was assayed by adding 0.5mL of native invertase in 0.1 M acetate buffer, pH 4.5 to 2.5 mL of sucrose (60 mg/mL) in the same buffer. After exactly 3 and 6 min. of incubation at 50°C, then 10µL sample was taken and the released glucose was determined by conventional tests (Glukoza OXY DST). The absorbance at 500 nm, (HITACHI U-2800A) was measured and recalculated using the glucose standard absorbencies (15 mmol). The enzyme activity unit (U) was defined as the amount of enzyme liberating 1 mmol of glucose per minute under the assay conditions.

Protein concentration was determined spectroscopically at λ= 280 nm and/or by Lowry’s method.

2.5.2 Activity of Immobilised Invertase

Immobilized enzyme preparation (0.05–0.1 mL) in 4 mL of acetate buffer, pH 4.5 was placed into a stirred reactor (250 rpm, 50°C), then 20 mL of preheated substrate (60 mg/mL) was added. After 3, 6, 10 and 20 min, 10µL of suspension was taken and glucose concentration was determined by conventional tests (Glukoza OXY DST). The absorbance at 500 nm, (HITACHI U-2800A) was measured and recalculated using the glucose standard absorbencies (15 mmol). The enzyme activity unit (U) was defined as the amount of enzyme liberating 1 mmol of glucose per minute under the assay conditions

3 RESULTS AND DISCUSSION

3.1 Properties of Modified Carriers

<table>
<thead>
<tr>
<th>Carriers</th>
<th>S_BET [m²/g]</th>
<th>V_pN2 [cm³/g]</th>
<th>d_p [nm]</th>
<th>d_w [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBA-15S</td>
<td>645.19</td>
<td>0.71</td>
<td>6.3</td>
<td>-</td>
</tr>
<tr>
<td>SBA-15S-A</td>
<td>316.86</td>
<td>0.44</td>
<td>5.5</td>
<td>-</td>
</tr>
<tr>
<td>SBA-15L</td>
<td>634.62</td>
<td>2.16</td>
<td>25.2</td>
<td>10.7</td>
</tr>
<tr>
<td>SBA-15L-A</td>
<td>395.81</td>
<td>1.58</td>
<td>25.0</td>
<td>9.6</td>
</tr>
<tr>
<td>MCF</td>
<td>622.37</td>
<td>2.46</td>
<td>28.6</td>
<td>14.6</td>
</tr>
<tr>
<td>MCF-A</td>
<td>349.46</td>
<td>1.8</td>
<td>28.3</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Table 1: Structure parameters of carriers.

Nitrogen adsorption-desorption isotherms for the carriers used prior to their modification are shown in Figure 1. They are all of type IV and all exhibit a clear hysteresis loop at relative pressure from 0.6 to 0.9. The sharp rise in the nitrogen adsorption–desorption isotherms at very high relative pressures indicates the presence of large mesopores and even small macropores. As shown in Table 1 the modification of pristine MS with organosilanes resulted in a small decrease in values of all texture parameters. Decrease of mesopore volumes (V_pN2) somewhat larger for SBA-15S than for SBA-15L and MCF, and also decrease in the value of specific surface area.
Decrease in these parameters had notably less effect on the size of caverns and windows.

3.2 Invertase Based Catalysts

Invertase immobilization on aminated MS was carried out at pH = 6, that was found an optimal value for the covalent invertase bonding. From practical point of view the effective, (measured) activity of biocatalysts is the most important property. It is affected by both the amount of proteins bound with a carrier, their intrinsic activity and the accessibility of active sites to the substrate. The results of experiments carried out with unmodified carriers (not shown here) indicated a total lack of enzymatic activity despite a notable enzyme adsorption. In the case of protein adsorption on aminated MSs (Figure 2) we observed that the enzyme loading strongly depends on pore size. For SBA-15S-A it was only 0.11 mg/mL of carrier, whereas for SBA-15L-A and MCF-A where it is about three times more. The same tendency was also observed in the biocatalysts activity. When invertase was anchored covalently (Figure 3) protein load in SBA-15S-A and SBA-15L-A was about twice and for MCF-A even fivefold larger than for the samples with invertase attached by physical adsorption with subsequent crosslinking of proteins. However, this significant increase in the protein loading resulted in the activities of the corresponding catalysts, that were by factors of about 17, 11 and 27 larger, and that is quite amazing. This clearly indicates that covalent bonding via amino groups with GLA activation is exceptionally effective for invertase immobilization. That may be explained by the most favorable environment formed jointly by ultra-large, cage-like silica mesopores, the surface of which was modified with amino groups and further with GLA. Such microenvironment was lacking in unmodified carriers. As we can observe (Figure 3) the aminated MCF bounded more proteins and had better activity than the corresponding SBA-15L catalysts, despite quite similar values of surface area, pore volume and pore diameter. This may be explained by smaller window diameters seen in SBA-15L than MCF, that hinder mass transport.

Figure 1: Nitrogen adsorption isotherms of (A) MCF, (B) SBA-15L, (C) SBA-15S.

Figure 2: Performance of invertase physically adsorbed on aminated MS with subsequent crosslinking of enzymes. Protein loading -grey; activity-black.

Figure 3: Performance of invertase covalently attached to various MS. Bound protein-grey; activity-black.
The preferential binding of selected proteins with carriers can be evaluated from the values of specific activities of the native enzyme in the eluates. As can be seen in Figure 4, the specific activities of invertase in the eluates, obtained after its adsorption on aminated MS, are higher than observed for the native enzyme. This indicates the preferential sorption of ballast proteins onto the silica matrix and slightly higher wash out of enzyme proteins. In the case of covalent bonding of invertase (Figure 5) there is no significant difference between the specific activities of native invertase and the eluates. Moreover, the specific activity of anchored invertase is very similar to that of a native enzyme, and it indicates that no significant lose in activity during the covalent immobilization procedure occurred.

The storage stability of enzyme preparations is also of practical importance. All the biocatalysts obtained by physical adsorption on aminated silicas showed about 95-105 % storage stability, while for those obtained by the covalent method it is about 85-95%. And that is also quite attractive result.

4 CONCLUSIONS

Siliceous mesostructured cellular foams with invertase covalently anchored to their surface by glutaraldehyde-amino linkage show very attractive biocatalytic properties, far superior to the corresponding SBA-15-based biocatalyst. In the case studied, regardless the carrier, the covalent attachment of enzymes appeared to be far more effective immobilization method than the two adsorption-based methods with the additional protein crosslinking, proposed most recently. These observations contradicts a common view that the harsh conditions of proteins’ anchoring by covalent bonds strongly denature enzymes’ superstructure and hence reduce their activity.

Acknowledgement

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