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# Covalent attachment of cyclodextrin glucanotransferase from genetically modified *Escherichia coli* on surface functionalized silica coated carriers and magnetic particles

## ABSTRACT

Recombinant cyclodextrin glucanotransferase JCGT8-5 (CGTase, EC 2.4.1.19) was effectively immobilized by formation of chemical bounds on polyethylenimine (PEI)-activated silica particles, on silanized Spherosils 5, XOA200, XOC 005, Arylamine CPG and magnetite $Fe_3O_4$ ($Fe(II)O.Fe(III)_2O_3$) micro-particles and by adsorption on magnetite/activated carbon support. The immobilization capacities of the carriers were determined. Binding yields reached from 50% to 93%. The amount of bound enzyme was considerably higher when this protein was immobilized on polyethylenimine (PEI)-activated silica particles and silanized Spherosil 5, XOA and XOC micro-particles and reached 1.33-1.77 mg/g support. Arylamine CPG and the activated carbon/magnetite support also possessed high binding capacities – 0.73-0.78 mg enzyme/g carrier. Analysis of formed CDs revealed changes in the β-cyclodextrin: γ-cyclodextrin ratio due to immobilization.

**Key words:** Immobilization, recombinant CGTase, magnetic and silica carriers, products ratio

## Introduction

Enzyme immobilization greatly simplifies the whole bioprocess because the immobilized enzyme can be recovered and reused again in the subsequent reaction cycle, as long as the enzyme still maintains its functionality and operational stability. The choice of the immobilization method depends on the enzyme stability (Kelly et al., 1977; Gemeiner, 1992) and the process of application. The most commonly used carrier binding methods are adsorption or covalent linkage to a carrier (Kierstan & Coughlan, 1991; Gemeiner, 1992; Katchalski-Katzir & Kraemer, 2000) because in the protein (enzyme) molecule a number of amino acid functional groups are suitable for covalent bond formation. Those that are most often involved are the amino group of lysine or arginine, the carboxyl group of aspartic and glutamic acids, the hydroxyl group of serine or threonine and the sulfhydryl groups of cycteine. Silica micro- and nanoparticles provide many surface reactive groups, substantially vi a silanol or Si–OH groups, to be directly employed in subsequent surface functionalization (Kierstan & Coughlan, 1991). Surface modification by silanization is very common method for particle functionalization. High density of free amino groups ($-NH_2$) lying outwards the particle surface provides an excellent media for further chemical surface modification such as enzyme cross-linking with glutaraldehyde.

In the recent years, an increased attention is particularly focused on production of functionalized magnetic micro- and nanoparticles (MMPs and MNPs) for uses in immobilization of various bioactive compounds such as proteins, DNA, various chemicals, viruses - key components in different areas like catalysis, environmental remediation, the biomedical field and sensing devices, cell labeling and immunomagnetic separations, cell isolation, drug targeting, waste water treatment and other applications (Šafařik & Šafařikova, 1999, 2002; Häfeli, 2001; Berensmeier, 2006; Luo et al., 2006; Wu et al., 2008; Stanciu et al., 2009; Šafařik et al., 2011). Iron oxides, mainly $Fe_3O_4$ have been the most widely used because of their simple preparation and

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superparamagnetic properties (Majewski & Thierry, 2008). Fabricating biofunctionalized magnetic materials containing a high amount of the biological element with high activity and stability is essential for their application (Stanciu et al., 2009). Various routes for the fabrication of biofunctionalized magnetic micro- and nanoparticles include traditional methods such as covalent binding, adsoprtion, specific affinity interactions, and entrapment in porous surface layers (Wang & Lee, 2003). Functionalization of magnetic particles with carboxyl, amino or hydroxyl groups prior to the covalent binding is traditionally used (Zhang et al., 2002; Yamaura et al., 2004; Liang et al., 2007; Hanessian et al., 2008; Majewski & Thierry, 2008; Murbe et al., 2008; Yong et al., 2008; Forge et al., 2011).

Enzymes covalently immobilized on the surface of magnetic particles include glucose oxidase (Rossi et al., 2004) and peroxidase (Wei et al., 2007), as well as cholesterol oxidase, lipase (Dyal et al., 2003), trypsin and chymotrypsin (Hong et al., 2006; 2007; Zavisova et al., 2006; Li et al., 2007a; 2007b; Ivanov et al., 2009), CGTase (Ivanova, 2010; Ivanova et al., 2010), chitosanase on amylose coated $Fe_3O_4$ magnetic nanoparticles (Kuroiwa et al., 2008).

In the present study, magnetite microparticles, silicates such as Spherosils 5, XOA 200, XOC 005, and Arylamine CPG additionally functionalized with amino silane were prepared for recombinant CGTase JCGT8-5 covalent coupling. Immobilization on PEI-activated silica beads and adsorption on activated carbon support, containing magnetite, were also studied. Up to now there is not information about immobilization of recombinant CGTase from alkaliphilic halotolerant strain on such particles.

## Materials and Methods

### *Enzyme*

Cyclodextrin glucanotransferase (CGTase, EC (EC 2.4.1.19) gene from an alkaliphilic halotolerant *Bacillus pseudalcaliphilus* 8SB strain was identified and sequenced. It was cloned as a PCR amplicon, introduced and expressed in *E. coli* BL21 (DE3) recombinant pJCGT8-5 (Petrova et al., 2012). The synthesized CGTase is a monomer with a molecular mass 75.5 kDa and produces only two types of cyclodextrins (β- and γ-CD). Recombinant CGTase JCGT8-5 produced 83% β-CD and 17% γ-CD after 20 min reaction time as the native crude enzyme after 24 h reaction time (Atanasova et al., 2008). In respect to the purified native enzyme this ratio was 82% β-CD and 18% γ-CD (Kitayska et al., 2011). Semi-purified enzyme concentrate with activity 5000 U/ml and specific activity 3180 U/mg protein was used without further purification.

### *Supports for immobilization*

Several silica and magnetite supports were used:

**A** - Poly-(ethyleneimine)-activated silica (Sigma-Aldrich), 20-40 mesh (0.84-0.35 mm), pore diameter 250Å (25 nm) (*PEI-silica*) and poly-(ethyleneimine)-activated silica, benzylated beads (Sigma-Aldrich), 20-40 mesh, (0.84-0.35 mm) (*Benz-PEI silica*). These carriers were used for immobilization after treatment with glutaraldehyde.

**B** - Arylamine CPG, porous glass beads, 200-400 mesh (0.076-0.035 mm) (Sigma-Aldrich); - Porous silica particles – Spherosil 5 (IBF Biotechnics, France), surface 5 $m^2$/g, pore diameter 460 nm (4600 Å); – Spherosil XOA 200 (Rhône-Poulenc, France), 0.20-0.35 mm, surface 125-250 $m^2$/g, pore diameter 100-200 Å (10-20 nm); – Spherosil XOC 005 (Rhône-Poulenc, France), 2-6 $m^2$/g, pore diameter >1500 Å (>150 nm). These particles were treated with (3-aminopropyl)-triethoxysilane and glutaraldehyde.

**C** – Magnetite microparticles (magnetite $Fe_3O_4$ ($Fe(II)O.Fe(III)_2O_3$), 100-120 μm) – silanized and activated with glutaraldehyde (*SMM*).

**D**– Non-porous magnetite particles (*NPMP*, magnetite $Fe_3O_4$) covered with epoxy resin (condensation of epichlorhydrin with bisphenol) and with second layer from activated carbon, 1.0-1.2 mm diameter) (Ivanova et al., 1996).

### *Support activation*

PEI-silica, Benz PEI-silica, the Spherosils, Arylamine CPG and the magnetite microparticles (SMM) were silanized applying a similar procedure, as described earlier (Ivanova et al., 2010). They were treated 4 h with 3-10 ml/g support of 5.0% (v/v) solution in acetone of (3-aminopropyl)-triethoxysilane (Aldrich) at 50°C, followed by washing step with acetone, ethanol, distilled water and 0.1 M phosphate buffer, pH 6.0. Carbonyl derivatives were obtained after 4 h of reaction with 2.5% (v/v) glutaraldehyde (Fluka) at room temperature and then were flushed with 0.5 litter/gram distilled water and 0.1 M phosphate buffer, pH 6.0 and stored at 4°C.

### *Immobilization*

Unless otherwise stated, 2.5 ml of 0.1 M phosphate buffer, pH 6.0, containing 0.1-2.25 mg of recombinant

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CGTase as protein, was added to 100 mg of support at 4°C. Resulting mixture was stirred for 4-24 h. After the coupling step, the supernatant was removed with pipette, the supports were washed with water and buffer until no protein was detectable in the filtrates. The enzyme-carrier complex was stored at 4°C in 0.1 M buffer, pH 6.0.

***Magnetite/activated carbon carrier – preparation, adsorption and crosslinking***

Particles of commercial catalyst "Haldorf Topsoe" KM-1 (Denmark) for ammonia synthesis were used as magnetic cores. They were covered with a stable film of epoxy resin obtained by condensation of epichlorhydrin with bisphenol as described (Ivanova et al., 1996). One kg of the catalyst (sieve diameter 0.8-1.0 mm) was mixed with 100 g of the epoxy resin and with 9.0 g 1,6-hexanediamine. After 10 min, the magnetic cores were covered with a thin film; then activated carbon powder (Fluka Chemie AG, Buchs, Switzerland, DAB powder, particle size 78% < 40 μm, bulk density-495 kg/m$^3$) was added in excess and mixed slowly in a drum for 15 min. During this operation a layer of powder on the resin film was formed. The beads were dried for 48 h at room temperature to complete the resin solidification. The final step was particle washing and drying. The final bead diameter was in the range from 1.0 to 2.0 mm (the average diameter was 1.2 mm). The shape was near spherical. The supports were treated consecutively with 1% NaOH and HC1 and were washed with distilled water to neutral pH before the immobilization. After sterilization for 3 h at 105°C, 100 mg of beads were mixed for 4-24 h at 25°C with 0.5-1.5 mg recombinant enzyme per g of support. Then glutaraldehyde (2.5% solution) was added. After the crosslinking process (30 min), the particles were washed with distilled water and phosphate buffer to remove the excess of enzyme and glutaraldehyde.

***Determination of the amount of bound enzyme***

The amount of protein loaded onto the supports was according to the Bradford assay (Bradford, 1976). Immobilized protein quantity was measured after each washing step at 595 nm. Bovine serum albumin (BSA) was used as a standard protein.

***Determination of formed cyclodextrins***

Formation of cyclodextrins from soluble starch was determined as follows: - 100 mg of carriers with immobilized recombinant CGTase were incubated 20 min at 60°C in 1.0 ml 0.1M phosphate buffer, containing 4% soluble starch (Fluka). The reaction was stopped by placing the samples in a boiling water bath for 15 min. Starch hydrolysates were chilled, passed through a Millipore filter 0.2 μ and analyzed for CDs by HPLC (system Waters equipped with RI Detector 2414, column YMC-Pack-ODS-AQ (150 mm×4.6 mm) at 30°C. The mobile phase was methanol/water (7:93, v/v) with flow rate of 1.0 ml/min. Injection volume was 20 μl. Standards comprised α-, β- and γ– cyclodextrins (Fluka).

## Results

***Immobilization yield***

The recombinant enzyme was immobilized on eight carriers by covalent linking via glutaraldehyde to silanized silica and magnetite particles and adsorbed and cross-linked on magnetite/activated carbon support. The results for the immobilization efficiency as a function of the CGTase quantity in the solution for immobilization and the reaction time are summarized in Tables 1 and 2.

The quantity of bound CGTase reached 0.23-1.77 mg immobilized protein per gram carrier. The amount of immobilized enzyme increased with increasing enzyme concentration during the immobilization step. However higher binding yields (BY) were reached at the lowest protein concentration – 0.1 - 0.5 mg, for all studied carriers, except the PEI silica beads (Table 1). The highest results for BY were obtained with aminosilane modified Spherosils 5, XOA 200 and XOC 005 (89-98% of the added protein). Similar results were obtained after adsorption/cross-linking process on magnetite/activated carbon particles (89-94%). Silanized magnetite (SMM) is a carrier on which binding yields from 50 to 90% were measured, but these results were obtained with the lower initial protein concentrations (0.1-0.4 mg/g support) and the immobilized enzyme quantity on this support was also lower (Table 2). The fixation of recombinant CGTase on Spherosil XOA 200 was the most effective one – 1.77 mg bound protein/g support, followed by the other Spherosil particles – XOC 005 and Spherosil 5 – 1.62 - 1.33 mg/g carrier.

The effect of enzyme coupling time on recombinant CGTase immobilization was investigated. It was found that the CGTase JCGT8-5 immobilization onto aldehyde-functionalized PEI silica particles proceeded within 4 hours with binding efficiency (BY) 67-88%. Further prolongation of the immobilization led to decrease of the binding yield. Comparing the results from Tables 1 and 2 it could be concluded that at initial enzyme concentration of 1.5 mg/g carrier some of the supports were saturated and the amount of

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fixed enzyme was unaffected by the reaction time – these are the Arylamine CPG and the magnetite/activated carbon maximal binding capacity at enzyme concentration 0.4 mg/g carrier. All other support needed more than 4 hours for the immobilization and the highest binding yields were obtained after 24 h at 4°C.

The experimental results show that the structure and the great number of amino groups per gram of support (in the case of PEI-treated support) lead to faster immobilizing of CGTase.

Other factor, affecting the process is the particle surface area. Higher quantity of enzyme was immobilized on Spherosil XOA 200 with surface area 125-250 $m^2/g$. Spherosil 5 and Spherosil XOA 005 have a similar surface area – 5 $m^2/g$ and 2-6 $m^2/g$, respectively, but better results were obtained with the Spherosil XOC 005 – 1.62 mg/g, possessing lower pore diameter than the Spherosil 5. It's very likely that the enzyme can not be immobilized inside the support pores due to their small diameter. However, the big surface of the carrier increases its immobilization capacity.

particles. The silanized magnetite (SMM) reached its

***Starch conversion to CDs and product ratio***

Maximal starch conversion was obtained with immobilized on Spherosil 5 (3.5-9.1%) and Spherosil XOC 005 (3.6-5.1%) recombinant CGTase.

**Table 1.** *Immobilization yield as function of the immobilization time and the added protein.*

| **Carrier/ Immobilization time, h** | | **Immobilization yield, %** *Initial protein added, mg/g carrier* | | |
|---|---|---|---|---|
| | | *0.5* | *1.0* | *1.5* |
| **PEI silica** | **4** | 67.6 | 84.4 | 88.4 |
| | **8** | 55.9 | 70.4 | 84.0 |
| | **24** | 55.1 | 60.4 | 70.9 |
| **Benz PEI silica** | **4** | 70.7 | 69.9 | 69.9 |
| | **8** | 80.1 | 68.7 | 68.1 |
| | **24** | 79.1 | 72.8 | 70.5 |
| **Arylamine CPG** | **4** | 74.2 | 54.9 | 48.7 |
| | **8** | 68.7 | 53.5 | 42.8 |
| | **24** | 66.9 | 57.6 | 41.5 |
| **Spherosil 5** | **4** | 91.8 | 90.3 | 81.6 |
| | **24** | 94.3 | 86.5 | 88.8 |
| **Magnetite/activated carbon, NPMP** | **4** | 89.1 | 58.1 | 52.0 |
| | **24** | 94.3 | 55.1 | 51.4 |
| | | *Initial protein added, mg/g carrier* | | |
| | | *0.75* | *1.5* | *2.25* |
| **Spherosil XOA 200** | **4** | 81.9 | 69.5 | 61.2 |
| | **24** | 89.5 | 83.1 | 78,8 |
| | | *Initial protein added, mg/g carrier* | | |
| | | *0.69* | *1.38* | *2.07* |
| **Spherosil XOC 005** | **4** | 79.8 | 65.3 | 65.3 |
| | **24** | 98.0 | 81.2 | 78.2 |
| | | *Initial protein added, mg/g carrier* | | |
| | | *0.1* | *0.3* | *0.4* |
| **Magnetite, SMM** | **4** | 50.4 | 43.6 | 50.6 |
| | **24** | 90.7 | 77.1 | 51.0 |

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**Table 2.** *Maximal protein loading of the carriers.*

| Support | Added enzyme (mg/g support) | Immobilized enzyme quantity (mg/g support) | Optimal time (h) |
|---|---|---|---|
| **PEI silica** | 1.50 | 1.33 | 4 |
| **Benz PEI silica** | 1.50 | 1.06 | 24 |
| **Arylamine CPG** | 1.50 | 0.73 | 4 |
| **Spherosil 5** | 1.50 | 1.33 | 24 |
| **Spherosil XOA 200** | 2.25 | 1.77 | 24 |
| **Spherosil XOC 005** | 2.07 | 1.62 | 24 |
| **Magnetite** | 0.3 | 0.23 | 24 |
| **Magnetite/activated carbon** | 1.50 | 0.78 | 4 |

Analysis of formed CDs revealed changes in the β-cyclodextrin: γ-cyclodextrin ratio due to immobilization. Recombinant native CGTase JCGT8-5 produced 83% β-CD and 17% γ-CD and the native purified original enzyme 82% β-CD and 18% γ-CD. The fixed recombinant CGTase produced only β- and γ-CDs from starch similarly to both native enzymes.

The ratio between the two CDs depended on the enzyme quantity fixed, on the enzyme in the fixation solution and in some cases on the fixation time. Comparing the results on Tables 2 and 3 for the Spherosils it is evident that the best starch conversion to CDs was obtained with recombinant CGTase immobilized on the Spherosil 5 while the best protein fixation was on Spherosils XOA 200 and XOC 005. Similar were the results for the PEI silica and Benz PEI silica. The lower the fixed enzyme quantity was, the higher the conversion was (for initial protein quantity 1.5 mg/g carrier).

Also it could be seen that the fixed enzyme quantity on each carrier affected the β-: γ-cyclodextrin ratio. The lower the immobilized recombinant enzyme amount was, the lower the ratio becomes and higher amounts of γ–cyclodextrin were formed. For example, the ratio between the produced CDs by the immobilized on PEI silica enzyme was 80:20 at the lower fixed enzyme quantity and 82:18 at higher quantity. After fixation on Spherosil 5 these ratios were 82:18 and 84:16, respectively. Similar were the results for the preparations on Benz PEI silica, Arylamine CPG, Spherosil XOA 200 - 84:16; 83:17; 85:15, etc.

Further information about the possibility for biotechnological magnetical or nonmagnetical application in the synthesis of cyclodextrins of the fixed on solid magnetic supports or in porous silica carriers recombinant CGTase JCGT8-5 will be obtained after additional study of immobilized enzyme storage and continuous operational stabilities.

**Table 3.** *Cyclodextrin production by immobilized crude recombinant enzyme.*

| Protein for fixation, mg/g carrier | Fixation time, hours | γ-CD, mg/ml | β-CD, mg/ml | total CDs, mg/ml | Starch conversion into CD, % | Ratio β-:γ-CD, % |
|---|---|---|---|---|---|---|
| | | | *PEI silica* | | | |
| 0.5 | 8 | 0.175 | 0.738 | 0.913 | 2.3 | **80.8:19.2** |
| 0.5 | 24 | 0.218 | 0.861 | 1.079 | 2.7 | **79.8:20.2** |
| 1.0 | 8 | 0.205 | 0.913 | 1.118 | 2.8 | 81.7:18.3 |
| 1.0 | 24 | 0.330 | 1.587 | 1.917 | 4.8 | 82.8:17.2 |
| 1.5 | 8 | 0.234 | 1.083 | 1.317 | 3.3 | **82.2:17.8** |
| | | | *Benz PEI silica* | | | |
| 1.5 | 8 | 0.298 | 1.577 | 1.875 | 4.7 | **84.1:15.9** |

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| Protein for fixation, mg/g carrier | Fixation time, hours | γ-CD, mg/ml | β-CD, mg/ml | total CDs, mg/ml | Starch conversion into CD, % | Ratio β-:γ-CD, % |
|---|---|---|---|---|---|---|
| | | | *Arylamine CPG* | | | |
| 1.5 | 7 | 0.250 | 1.226 | 1.476 | 3.7 | **83.1:16.9** |
| | | | *Spherosil 5* | | | |
| 0.5 | 4 | 0.248 | 1.140 | 1.388 | 3.5 | 82.1:17.9 |
| 0.5 | 24 | 0.252 | 1.161 | 1.413 | 3.5 | 82.2:17.8 |
| 1.0 | 4 | 0.380 | 1.917 | 2.297 | 5.7 | 83.5:16.5 |
| 1.0 | 8 | 0.432 | 2.204 | 2.636 | **6.6** | **83.6:16.4** |
| 1.0 | 24 | 0.431 | 2.206 | 2.637 | **6.6** | **83.7:16.3** |
| 1.5 | 4 | 0.530 | 2.562 | 3.092 | **7.7** | **82.9:17.1** |
| 1.5 | 24 | 0.588 | 3.057 | 3.645 | **9.1** | **83.9:16.1** |
| | | | *Spherosil XOA 200* | | | |
| 0.75 | 4 | 0.179 | 0.739 | 0.918 | 2.3 | **80.5:19.5** |
| 0.75 | 24 | 0.129 | 0.563 | 0.692 | 1.7 | 81.4:18.6 |
| 1.5 | 4 | 0.156 | 0.782 | 0.938 | 2.3 | 83.4:16.6 |
| 1.5 | 24 | 0.218 | 1.060 | 1.278 | 3.2 | 82.9:17.1 |
| 2.25 | 4 | 0.148 | 0.708 | 0.856 | 2.1 | 82.7:17.3 |
| 2.25 | 24 | 0.197 | 1.117 | 1.314 | 3.3 | **85.0:15.0** |
| | | | *Spherosil XOC 005* | | | |
| 0.69 | 4 | 0.201 | 0.971 | 1.172 | 2.9 | 82.8:17.2 |
| 0.69 | 24 | 0.306 | 1.482 | 1.788 | 4.5 | 82.9:17.1 |
| 1.38 | 4 | 0.295 | 1.446 | 1.741 | 4.3 | 83.1:16.9 |
| 1.38 | 24 | 0.326 | 1.600 | 1.926 | 4.8 | 83.1:16.9 |
| 2.07 | 4 | 0.236 | 1.190 | 1.426 | 3.6 | 83.5:16.5 |
| 2.07 | 24 | 0.357 | 1.698 | 2.055 | 5.1 | 82.6:17.4 |
| | | | *Magnetite/activated carbon* | | | |
| 0.5 | 8 | 0.063 | 0.284 | 0.347 | 0.87 | 81.8:18.2 |

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