
Immobilization of recombinant inulase II from a genetically modified *Escherichia coli* strain

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Abstract

In order to characterize the immobilization of an enzyme by adsorption onto a carrier, a considerable number of parameters are required. The resins investigated for the immobilization of E. coli inulase II are weakly basic anion-exchangers, having tertiary amines as functional groups. Calculating the activity of the immobilized enzyme, 2 out of the 4 tested resins proved to bind Inulase II from E. coli/pMSiftOptWT with acceptable efficiency. The results showed an acceptable efficiency of the immobilization on for Duolite A 568 and Amberlite 94 S, around 20% and 19,5%, respectively.

Keywords: enzyme immobilization, adsorption, ion-exchangers

Introduction

Enzymes are large protein molecules with chemically reactive groups, ionic groups, and hydrophilic as well as hydrophobic domains that can all participate in the immobilization process through physical adsorption, ionic binding, or covalent linkage. The immobilized enzyme is defined as “the enzyme physically confined or localized in a certain defined region of space with retention of its catalytic activity, which can be used repeatedly and continuously”[1]. An universal immobilization method does not exist. Each end use requires evaluation of the individual steps according to criteria such as the purpose of immobilization, activity, yield, stability, simplicity and economic feasibility. Each method has its advantages and disadvantages that have to be considered for every biocatalyst and enzymatic reaction investigated. The adsorption of an enzyme onto an insoluble support is the simplest method of enzyme immobilization. The major components of an immobilized enzyme system are the support (carrier), the enzyme and the mode of interaction of the enzyme with the support. The carrier may be a membrane, a water-insoluble solid, or a polymer matrix, classified according to their morphology (porous, nonporous, or gel type) and their chemical composition [2]

Types and concentration of carrier functional groups are essential characteristics with respect to enzyme immobilization. They determine the coupling reaction and the physico-chemical properties of the immobilized biocatalyst. Functional groups most often involved are carboxyl and amino groups. The anion-exchangers investigated in the frame of this paper are weakly basic resins with amino functional groups (see table 1).

Attachment of the enzyme to the carrier surface is governed by physical interactions such as van der Waals forces, hydrogen bonding, or hydrophilic-hydrophobic effects [3]. Although the biocatalyst may be immobilized without conformational changes associated with loss of activity, the binding is sensitive to environmental conditions, such as ionic strength, pH and temperature. A subsequent cross-linking step helps to stabilize the

immobilized biocatalyst on the carrier. Bi- or multifunctional reagents such as glutaraldehyde, toluene diisocyanate or bisdiazobenzidine derivatives can be used as cross-linking agents.

The yield of immobilized enzyme is used to characterize the immobilization efficiency and is defined as the ratio of immobilized protein based on enzyme offered for binding [4].

The adsorption of an enzyme onto an insoluble consists of mixing together the enzyme and the support material under certain conditions and then separating the two phases (soluble and insoluble). An important disadvantage of this method of immobilization is that the enzyme is not firmly bound to the support, so, to reduce the enzyme desorption, a cross-linking step is recommended (generally with glutardialdehyde) [5].

The immobilization is often not economical for cheap row-enzymes since the carrier and the immobilization price will not be compensated [6]. Compared to free enzymes, the immobilization of enzymes gives the possibility to use the immobilized biocatalysts either repeatedly, in batchwise reactions, or continuously, in tubular reactor systems. This repeated use leads to significantly lowered amounts of enzyme required for a certain biotransformation process. Further more, immobilized enzymes are often more stable than the free biocatalysts. Another advantage is that better and easier controlled processes can be performed and the biocatalyst can be easily recovered from the reaction mixture. These considerations were determining factors in seeking for an appropriate immobilization technique for *E.coli* Inulase II, involving a suitable resin.

Inulase II was produced as recombinant protein in genetically modified *Escherichia coli*. The *E.coli* XL-1 blue/pMSiftOptWT strain was obtained transferring the gene for inulase II from the natural producer, a strain of *Arthrobacter* spec., to *E. coli*. The inulase II was further used to obtain di-D-fructofuranose 1,2':2,3' dianhydride (DFA III) from inulin as a raw material [7].

Inulase II was immobilized until now, as reported in the literature, by covalent binding on porous glass [8], adsorption on bentonit or entrapping the whole cells in alginate [9] and entrapping the free enzyme in calcium alginate hydrogels [10]. In this paper, investigations on the adsorption of inulase II on anion-exchangers will be presented.

The resin charged with different anions was tested (OH^- , Cl^- and PO_4^{3-}) and also the influence of equilibration buffer pH was investigated. For OH^- ions, it was difficult to adjust the pH to the desired value and so phosphate buffer solutions with pH values in the range of 6 to 9 were used for further investigations on the resin equilibration.

E.coli/pMSiftOptWT inulase II enzyme solution had an activity of 3,500 U/L and it was investigated with respect to the adsorption behaviour with the following resins: Duolite A 561, Duolite A 568, Amberlite IRA 67, and Amberlite IRA 94 S (Fa. Rohm and Haas Deutschland GmbH, Frankfurt/ Main). All the investigated anion-exchangers are weakly basic resins and some parameters of these resins are summarized in table 1.

Table 1. Ion-exchangers basic parameters

Resin name	Structure size (μm)	Particle type	Matrix	Functional groups
Amberlite IRA 67	gel structure	500-750	acrylic-DVB*	-N-(R) ₂
Amberlite IRA 94 S	macroporous	350-470	styrene-DVB	-N-(R) ₂
Duolite A 561	macroporous	470-740	phenol-formaldehyde	-N-(R) ₂
Duolite A 568	macroporous	150-600	phenol-formaldehyde	-N-(R) ₂

* divinylbenzene

The pH range for immobilization was appropriately chosen to exclude the enzymes isoelectric point (for *Arthrobacter* sp. Inulase II = 4.5) (Walter, personal communication) considering that for pH values lower than the enzymes isoelectric point the cationic enzyme form is stabilized and so very poor adsorption onto an anion-exchanger resin should be obtained. A good adsorption onto the material investigated was expected for pH values higher than the isoelectric point, when the enzyme will be stabilized in overall anionic form. The activity of the immobilized enzyme increases with the buffer pH value (data not shown) and so resin equilibration with phosphate buffer, pH 9 was considered appropriate for further experiments.

Materials and Methods

The genotype of *Escherichia coli* XL-1 blue is: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F⁺ *proAB lacI*^qZ Δ*M15 Tn10* (Tet^r)].

The optimal cultivation parameters for this strain are : 37°C, 170 rpm, 16 hours, Luria-Bertani medium[11].

0,5 ml from the culture broth were centrifuged in a 10 ml glass test tube (Ø=13mm) using a Jouan Centrifuge (BR.4, Jouan Centrifuge, France) (rotor S40, 2,100 g, 10°C for 10 minutes). After centrifugation, the supernatant was removed by inverting the test tubes until all medium had been drained. Pellets were suspended in 5 ml of cold NaCl 0,9% (from ice) using a vortex device (dilution 1:10 regarding the native culture broth).

The cell suspension was then assayed for disruption, using an ultrasounds device (Sonopuls Homogeniser, Bandelin *electronic* GmbH & CoKG, Berlin, Germany) with the following parameters: (i) probe: KE76, (ii) cycle: 5×10%, (iii) power: 60% (iv) time: 2 minutes. The probe was not more than 2 cm immersed in the cell suspension, with no contact to the walls of the test tube. To prevent overheating, the test tubes were placed on ice during cell disruption. At the end, 2 ml from the disrupted cell suspension were further centrifuged in 2 ml Eppendorf cups for 5 minutes (Jouan centrifuge, rotor AB 2.14, 9,400 g, 4°C) and the supernatant was assayed for enzyme analysis.

Resin equilibration: the following resins were employed (Fa. Rohm and Haas Deutschland GmbH, Frankfurt/ Main): Duolite A 561, Duolite A 568, Amberlite 94 S, Amberlite IRA 67. 100 g resin and 1 L phosphate buffer (pH 9) were mixed and shaken (Edmund Bühler Swip KS 10) at room temperature for at least 4 hours (ratio resin: buffer = 1:10). The equilibrated resin was washed several times and could be stored for a long time in small amounts of distilled water.

Phosphate buffer: Na₂HPO₄: 35.6g/L; KH₂PO₄: 27.2g/L. The phosphate solutions were separately prepared and mixed in equal volumes. The pH of the obtained solution was around 6.7 and was adjusted to 9 with 10% NaOH.

Enzyme immobilization: 5 g equilibrated resin were mixed and shaken with 50 ml enzyme solution (ratio resin: enzyme = 1:10) for 24 hours at room temperature. In order to investigate the amount of unbound enzyme after immobilization, 1 ml from the supernatant was kept for further investigations. The supernatant was then decanted and the resin with immobilized enzyme was washed several times with dist. water.

Cross-linking with glutardialdehyde: the immobilized enzyme was shaken in a ratio 1:5 with 0.3% glutardialdehyde for 3 hours at room temperature. The supernatant was removed and the resin with immobilized enzyme was washed 4-5 times with dest. water. The immobilized enzyme was further assayed for enzyme analysis or stored in small amounts of water at 4°C.

Activity test for immobilized enzyme: 2 g immobilized enzyme and 20 ml inuline solution were shaken at 150 rpm for 30 minutes in a 50°C water bath. The enzymatic reaction was stopped boiling the samples (1 ml liquid sample, without resin) at 100°C for 5 minutes. 10 µl Novozym SP 230 were added (to hydrolyze the remaining inulin to fructose and glucose) and samples were incubated at 60°C for another 30 minutes. For desalting, ca. 150 mg ion-exchanger were introduced in every cup, and then were shaken at room temperature for 30 minutes (Edmund Buhler Swipe KS10). Samples were filtered through a 0.22 µl filter and measured by HPLC. The enzyme solutions had to be diluted with water for a final concentration of DFAIII in the reaction tube of around 3 g/L.

Inulin solution: 100 g inulin/L were dissolved in 0.04 M phosphate buffer pH 5.25 by heating the solution at 90-100°C under stirring. When the inulin was completely dissolved the solution was cooled down to 50-60°C using an ice bath. This solution was freshly prepared before use and will be further referred to as Inulin solution.

Phosphate buffer 0.04 M: 0.14 g/L Na₂HPO₄ × 2H₂O; 5.33 g/L KH₂PO₄ pH 5.25
The enzymatic activity of the enzyme is determined from the amount of DFAIII produced 1U of enzyme responds to 1µmol DFAIII per minute

The ion exchanger resins were obtained from Fa. Rohm and Haas Deutschland GmbH, Frankfurt/ and all the other chemicals were supplied by Merck, Darmstadt.

Results and discussions

To quantify the yield of immobilization, activity tests were performed for soluble (native) enzyme and for immobilized enzyme. Table 2. summarizes the immobilized activity and the immobilization yield obtained for the resins investigated. The values were calculated as percent of immobilized enzyme activity related to the free enzyme activity.

Table 2. Immobilization efficiency. The initial enzyme activity was 3,500 U/L for *E.coli/pMSiftOptWT.inulase II*

Resin	<i>E.coli/pMSiftOptWT inulase II</i>	
	Act _{imm.} (U/L _{resin})	Yield (%)
Duolite A 568	750	19.3
Duolite A 561	150	4.2
Amberlite 94 S	660	19.0
Amberlite IRA 67	56	1.6

The results presented in table.2. show that ~19.5% from the free enzyme activity was detected as immobilized activity when the enzyme was adsorpted on Duolite A 568, which corresponds to around 800 U/L_{resin}. When the immobilization was performed with Amberlite 94 S, around 650 U/L_{resin} immobilized activity was obtained, representing an immobilization yield of ~19%. The values obtained for Duolite A 568 and Amberlite 94 S make those two resins more suitable for further employment than Duolite A 561 and Amberlite IRA 67 which only bound up to 4.5% of enzyme.

To investigate the maximum loading of the resin, the enzyme solution was diluted with distilled water and assayed for immobilization on Duolite A 568. 5 g of equilibrated resin slurry were incubated at room temperature with 50 ml enzyme solution (pH 6.5) having different enzyme activities (volume ratio 1:10 between resin and enzyme solution). The enzyme activity was in the range of 1 to 300 U/ml. Duolite A 568 was further used for immobilization because in several experiments a higher activity than on Amberlite 94 S could be achieved. In order to calculate the immobilization yield, the activity of the immobilized enzyme was related to the enzyme activity employed for immobilization.

Activity tests were also performed to determine the amount of enzyme, which remained unbound after immobilization. Before cross-linking with glutaraldehyde, the liquid phase containing the unbound enzyme was separated from the immobilized enzyme by pouring off the supernatant. 100 μ l from this solution was tested for activity and the DFAIII concentrations obtained were calculated as activity of the unbound enzyme. The variations of immobilized activity and immobilization yield are presented in figure 1, plotted against the initial enzyme activity (in U pro ml enzyme solution).

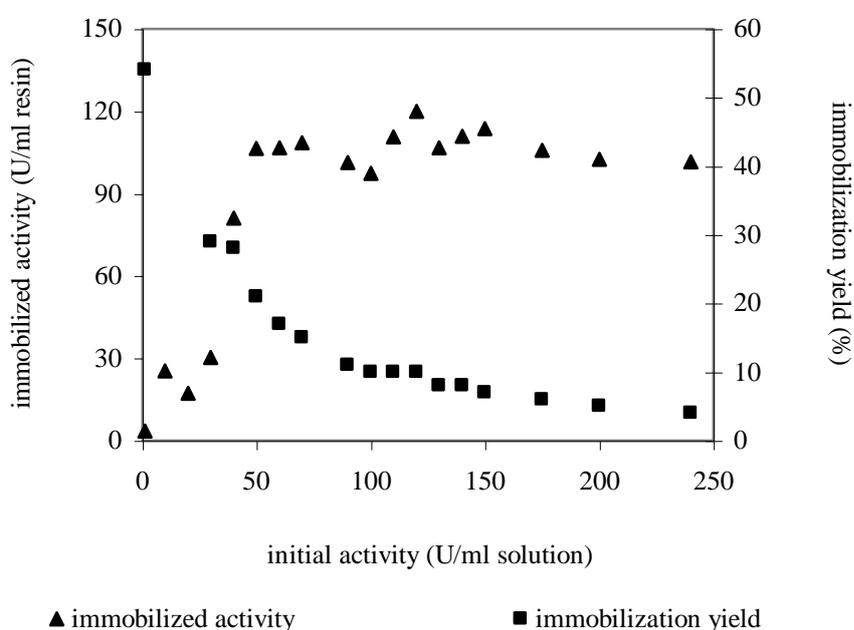


Figure 1. The correlated of immobilized activity and immobilization yield with the initial enzyme activity. The curves were obtained for *E.coli*/pMS*ift*OptWT enzyme immobilized on Duolite A568 and a ratio 1:10 of resin and enzyme solution.

The efficiency of the immobilization process decreases very rapidly with increasing initial enzyme activity. When only 1 U/ml_{solution} was used for immobilization, the immobilization efficiency is 54%, but the immobilized activity is very low. If the value for the immobilized activity remains constant around the maximum value of 110 U/ml_{resin}, it is natural that the immobilization process is less efficient with the increase of the enzyme concentration, which can be directly correlated with the enzyme activity. A maximal resin loading is reached when the immobilization is performed with a solution having an activity around 50 U/ml_{solution}. The efficiency of the process was calculated to be 20% for this solution. The free enzyme activity increases with the initial concentration of the enzyme employed for immobilization, but does not fit with the excess of enzyme when the maximum

loading of the resin was reached.. This lack of immobilized activity may be due to the crowding of other proteins on the support with a direct effect on the accessibility of enzyme molecules to the adsorption material [3]. The recombinant Inulase II from *E. coli*/pMSiftOptWT being an intracellular enzyme it is necessary to disrupt the cells in order to release it. No enzyme purification was performed before immobilization, so the enzyme molecules are competing with all the other host cellular proteins for the active functional groups of the resin.

On the other hand, when the immobilized activity remains constant with the increase of the enzyme loading on the support, one could expect to find an increasing amount of unbound enzyme. Still, the activity detected for the free enzyme does not fit an activity balance. For instance, when the immobilization was carried out with 240 U/ml_{solution}, the immobilized enzyme activity was 110 U/ml_{resin} and the immobilization yield was 5%. That means that only 5% enzyme bound to the resin and only 12% (instead of 95 %) could be detected as free enzyme (unbound) after immobilization. This important difference could possibly be explained by the fact that the enzyme molecules create overlapping layers surrounding the carrier particle. By standard activity tests, the exact quantification of the amount of enzyme involved in this immobilization process is not possible since, due to steric impediments, only the upper immobilized enzyme strata may participate in the enzymatic reaction.

To conclude, when immobilized on Duolite A 568, the maximum immobilized activity for *E. coli*/pMSiftOptWT inulase II was found to be around 110 U/ml_{resin}. Entrapping the *E. coli*/pMSiftOptRM inulase II in calcium alginate hydrogels, the immobilized activity reached was of 196 U/g [12]. For the cell-bound inulase II from *Arthrobacter ureafaciens* ATCC 21124 entrapped in calcium alginate, only an immobilized activity of 77 U/g was achieved [9]. Considering these results the adsorption on anion exchangers seems to be a more suitable immobilization method for inulase II.

In order to avoid the desorption under conditions to which the enzyme is subjected under reaction conditions (for instance pH, temperature, ionic strength), a subsequent immobilization step (cross-linking with glutardialdehyde) was performed. A thorough investigation on the cross-linking with glutardialdehyde was carried out by Monsan and co-workers [13]. Investigating the influence of glutardialdehyde over the glucoamylase activity, Nishimura *et al.* found that up to ~5.5%, the concentration of the cross-linking agent has no influence on the activity of the immobilized enzyme [5]. The immobilization was realized by covalent cross-linking of the enzyme with glutardialdehyde to an amino-group containing carrier (aminated silica gel) in the presence of a phenolic carboxylic acid (tanic acid) and a basic polysaccharide (chitosan). When the immobilization was made in the presence of glutardialdehyde, the enzyme activity was higher than the one obtained without cross-linking. The treatment with glutardialdehyde showed a 2-fold improvement in the stability of immobilized enzyme when the continuous saccharification of dextrin was performed, along with a 3-4-fold improvement in the heat stability. The Inulase of *Kluyveromyces fragilis* can be immobilized in the cells [14] by glutardialdehyde treatment. The behaviour of the enzyme in immobilized form is similar to the soluble one. Although the exact nature of the immobilization is not fully understood, no reduction of the enzyme activity was observed after glutardialdehyde treatment and the enzyme activity was not affected by the glutardialdehyde concentration. When the enzyme was entrapped in calcium-alginate

hydrogels [10], a glutardialdehyde concentration of 2.5 g/l showed not to affect the activity of the immobilized enzyme. 196 U/g were immobilized using increasing glutardialdehyde concentration and a reduction to 188 U/g (representing 80% from the initial activity) was registered when glutardialdehyde concentrations higher than 5 g/l were used. For the immobilization of the *E.coli* Inulase II on anion-exchangers resins, the cross-linking with a 3 g/l glutardialdehyde solution was considered appropriate and was shown not to lead to an enzyme deactivation.

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