***In vivo* chaperone assisted folding of α 1,6-fucosyltransferase
from *Rhizobium* sp.**

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Glycosyltransferases have become powerful tools for the synthesis of oligosaccharides through their strict control over the stereo- and regioselectivity of glycosidic bond formation.^[1] One major drawback of glycosyltransferases in synthesis is their limited availability. The cloning and over-expression of bacterial glycosyltransferases^[2] is one alternative to overcome this problem. However, when a heterologous protein is over-expressed in *E. coli* misfolding and aggregation happen frequently, driving the recombinant protein into inactive aggregates known as inclusion bodies (I.B.). One possible and attractive strategy to avoid their formation is to increase the cellular levels of molecular chaperones.^[3] Chaperonins are able to mediate ATP-dependent folding of polypeptides to the native state.^[4] GroEL from *E. coli* is the best characterized chaperonin and its function is dependent on the cochaperonin GroES.^[5]

Once that the recombinant enzyme is successfully produced, it is necessary to face the downstream process for its purification. When the recombinant enzyme preparation is to be used as biocatalyst, one affinity chromatographic step can give the required purity degree. Immobilized Metal Ion Affinity Chromatography (IMAC)^[6] can also be used for enzyme immobilization,^[7] since the adsorption of the protein to the chromatographic resin is not by the enzyme active center.

Recently we have over-expressed the enzyme α 1,6-fucosyltransferase (α 1,6-FucT) from *Rhizobium* sp. in *E.*

coli.^[8] In this heterologous system the enzyme was mainly expressed as I.B's. Several attempts to *in vitro* refold the recombinant enzyme were unsuccessful. Additionally, the enzyme expressed in soluble form shown a poor stability mainly due to enzyme precipitation. The *Rhizobium* sp. α 1,6-FucT have a transmembrane-like region of 19 aa close to the C-terminal of the enzyme. The influence of this region on the misfolding and instability of the recombinant α 1,6-FucT was demonstrated in a deletion mutant where a C-terminal fragment of 65 aa, including the transmembrane-like region, was removed.^[9]

In this work we have faced the optimization of the production, purification and stability of the wild-type α 1,6-FucT from *Rhizobium* sp. Thus, we have studied the effect of coexpress the molecular chaperone system GroEL/GroES on the yield of soluble recombinant enzyme, the one step purification/immobilization of the His tagged α 1,6-FucT on Ni²⁺-IDA-agarose and the activity and stability of the immobilized enzyme in the fucosylation reaction of chitobiose.

To increase the cellular levels of molecular chaperones, the plasmid pAG^[10] bearing *groEL/groES* genes was used to cotransform XL1Blue-MRF' cells along with pKK1,6FucT.^[8] The supplementation of GroEL/GroES strongly increased the correct folding of the transferase. Thus, the fucosyltransferase activity per liter of culture found soluble (6.0 U/L) was almost twice than that found when pKK1,6FucT was expressed alone (Table 1). The effect of over-express the chaperonins

GroEL/GroES per unit of biomass is even higher since the final cellular density reached by the culture was about 30% lower (Table 1).

For the purification of the soluble α 1,6-FucT we tried an approach based on the use of dye-affinity systems.^[11] We were able to obtain the recombinant enzyme with a high purity degree, but the enzyme was precipitated and inactive after elution from the column. In view of these results, we decided to sub-clone the α 1,6-FucT in the pRSET plasmid in order to get the fucosyltransferase tagged with a poly-Histidine tail in the N-terminal. The resulting pRSET α 1,6FucT plasmid was transformed into BL21(DE3) pLys cells and cotransformed, along with the pAG plasmid, in BL21(DE3) cells (Scheme 1). The expression of the α 1,6-FucT in BL21(DE3) pLys cells, was analyzed after induction with IPTG at 30 °C. The fucosyltransferase activity found soluble (1.3 U/L, Table 1), was lower than in the system pKK1,6FucT/XL1Blue-MRF'. However, the analysis of the expression by SDS-PAGE (Figure 1) showed that the total amount of recombinant enzyme expressed soluble as well as in I.B's, was much more higher than in the previous expression system. In the case of the BL21(DE3) cells, the increased level of intracellular chaperonins produced a significant increment in the soluble activity of α 1,6-FucT to 2.3 U/L (Table 1). In spite of the effect of the molecular chaperones, the major portion of the recombinant enzyme remained in the insoluble fraction. Our next approach to

optimize the yield of properly folded α 1,6-FucT was to decrease the growth temperature of the culture to 18 °C. It is well documented that lowering the cultivation temperature the protein aggregation is reduced.^[12] In our case, when α 1,6-FucT was expressed at 18 °C in absence of the pAG plasmid the production of soluble recombinant enzyme was not improved but decreased to 0.8 U/L, although the cell growth rate was also reduced (Table 1). When GroEL/GroES were coexpressed with the fucosyltransferase at 18 °C the soluble activity of α 1,6-FucT increased to 8.0 U/L (Table 1). This 10-fold increment was much more higher than the produced by the same chaperonins at 30 °C either when the α 1,6-FucT was expressed in the pKK223-3 plasmid or in the pRSET plasmid.

For the purification of the soluble histidine tagged α 1,6-FucT (H-T FucT) we used agarose gels with high density of Ni^{2+} (20-35 $\mu\text{mol Ni}^{2+}/\text{mL gel}$) immobilized through iminodiacetic acid (IDA) ligands. A crude preparation of H-T FucT was directly loaded on the Ni^{2+} -IDA-agarose column. In figure 2 can be observed that the H-T FucT was retained by the Ni^{2+} -IDA-agarose in a very selective way (Figure 2, lane 4). The immobilized α 1,6-FucT activity was 180-217 mU per g of gel with a specific activity of 75.6 mU per mg of protein. The stability of the immobilized H-T FucT was measured at 4 °C and compared with the stabilities of the enzyme purified and in a

crude preparation (Figure 3). The immobilized enzyme showed a strong stabilization with a half-life of 15 days.

Finally, the synthetic utility of the immobilized enzyme was proved in the fucosylation of chitobiose **2** (Scheme 2). The use of the immobilized enzyme present several advantages over the soluble one. Allows shorter reaction times because the higher concentration of the enzyme, the final yield (98 %) was higher and the work-up much more easier because the biocatalyst can be removed by a simple filtration.

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Legends of the Figures

Scheme 1. Construction of the pRSET1,6FucT and its transformation alone and with pAG plasmid. DNA manipulation was according to standard procedures.^[13] The *nodZ* gene was amplified by PCR using the oligonucleotides 5'-ATTCT**CTCGAG**ATGTACAATCGATAT-3' and 5'-ATATAG**GAATTCT**CAAGAGGCGGTATT-3' as leftward and rightward primers respectively (the recognition sequence for XhoI and Eco RI are in bold). GroEL/GroES expression was induced with L-arabinose (50 mg/L) when the cell growth reached an O.D. of 0.6. After 30 minutes the temperature was switched to 30 °C, or 18 °C when indicated, and the culture was induced with 1 mM IPTG to induce the expression of the α 1,6-FucT.

Scheme 2. Immobilized H-T FucT catalyzed fucosylation of *N,N'*-diacetylchitobiose (**2**) using GDP-Fucose (**1**) as donor. GDP-Fucose **1** (14.0 μ m, 6.0 mg) was incubated with chitobiose **2** (4.7 μ m, 3.0 mg) and Mn²⁺ (10 mM) in presence of the immobilized enzyme (0.7 g, 0.2 U/g gel) at 25 °C during 6 h.

Figure 1. SDS-PAGE^[14] analysis of the pKK1,6FucT and pRSET1,6FucT expression. Lane 1: molecular weight markers (rabbit muscle phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; chicken egg white ovalbumin, 45 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa); lane 2: soluble fraction of XL1-Blue MRF' cells harbouring the pKK1,6FucT plasmid; lane 3: inclusion bodies of XL1-Blue MRF' cells harbouring the pKK1,6FucT plasmid; lane 4: soluble fraction of BL21(DE3) pLys

cells harbouring the pRSET1,6FucT plasmid; lane 5: inclusion bodies of BL21(DE3) pLys cells harbouring the pRSET1,6FucT plasmid.

Figure 2. SDS-PAGE for the immobilization/purification of the H-T FucT. Lane 1: molecular weight markers as in figure 1; lane 2: CFE of BL21(DE3) cells harbouring the pRSET1,6FucT plasmid; lane 3: CFE after pass through Ni²⁺-IDA-agarose column; lane 4: Ni²⁺-IDA-agarose gel after his-tagged FucT immobilization.

Figure 3. Stability at 4 °C of the immobilized H-T FucT (●), purified H-T FucT (▲) and CFE of BL21(DE3) cells harbouring the pRSET1,6FucT plasmid (■). Immobilized fucosyltransferase was incubated in phosphate buffer (10 mM, pH 7.4) at 4°C without stirring. At different times aliquots were withdrawn and assayed for activity.

Table 1. Soluble activity of α 1,6-FucT in presence or absence of GroEL/GroES chaperonin system.

FucT/Chaperone	Induction <i>T</i> (°C)	Final O. D.	Activity (U/L) ^[a]
FucT	30	3.2	3.4
FucT/GroELS	30	2.2	6.0
His-Tagged FucT	30	3.1	1.3
His-Tagged FucT/GroELS	30	3.4	2.3
His-Tagged FucT	18	2.7	0.8
His-Tagged FucT/GroELS	18	2.4	8.0

^[a] The activity was measured at 25 °C for 20 min in a final volume of 0.3 mL, containing 12 mM Hepes (pH 7.7), 50 mM ClK, 6.5 mM MgCl₂, 0.7 mM PEP, 0.2 mM NADH, 6 U pyruvate kinase, 13 mU lactate dehydrogenase, 100 μ M of chitobiose and 52 μ M of GDP-Fucose. The assay was initiated upon addition of 10 mg of immobilized enzyme and the decrease in the absorbance at 340 nm was monitored.

Graphical Abstract

Coexpression of the chaperonins GroEL/GroES with the α 1,6 fucosyltransferase from *Rhizobium* sp. allows a 10-fold increment of soluble and active fucosyltransferase. The histidine-tagged fucosyltransferase can be purified and immobilized in only one step on Ni^{2+} -IDA-agarose gel. The immobilization produces an important stabilization of the recombinant enzyme, allowing the preparation of a robust biocatalyst for the synthesis of oligosaccharides.

Scheme 1

Scheme 2

Figure 1

Figure 2

Figure 3