In Vivo Chaperone-Assisted Folding of α-1,6-Fucosyltransferase from *Rhizobium* sp.

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chaperone proteins · gene expression · glycosyltransferases · immobilization · protein folding

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. One alternative to overcome this problem is the cloning and over-expression of bacterial glycosyltransferases[2]. However, when a heterologous protein is overexpressed in *E. coli*, misfolding and aggregation frequently occurs, which drives the recombinant protein into inactive aggregates known as inclusion bodies (IB). One possible and attractive strategy to avoid the formation of IBs is to increase the cellular levels of molecular chaperones.[3] Chaperonins are able to mediate ATP-dependent folding of polypeptides into the native folded state.[4] GroEL from *E. coli* is the best characterized chaperonin and its function is dependent on the cochaperonin GroES.[5]

Once the recombinant enzyme has been successfully produced, it is necessary to face the downstream process for its purification. If the recombinant enzyme preparation is to be used as a biocatalyst, the required degree of purity may be achieved by one chromatographic step. Immobilized metal ion affinity chromatography (IMAC)[6] can also be used for enzyme immobilization,[7] since the adsorption of the protein onto the chromatographic resin is not achieved through the enzyme active center.

Recently we overexpressed 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 IBs. Several attempts to refold the recombinant enzyme in vitro were unsuccessful. Additionally, the enzyme expressed in soluble form showed only poor stability, mainly because of enzyme precipitation. *Rhizobium* sp. α-1,6-FucT has a transmembrane-like region of 19 amino acids (aa) close to the C-terminus of the enzyme. The influence of this region on the misfolding and on the instability of the recombinant α-1,6-FucT was demonstrated with a deletion mutant in which a 65-aa C-terminal fragment including the transmembrane-like region was removed.[9]

Here we report the optimization of the production, purification, and stability of the wild-type α-1,6-FucT from *Rhizobium* sp. We have studied the effect of coexpression of 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 Ni^{2+}-IDA-agarose (IDA = iminodiacetic acid), and the activity and stability of the immobilized enzyme in the fucosylation reaction of chitobiose.

To increase the cellular levels of molecular chaperones, the plasmids pAG,[10] which contains the *groEL/groES* genes, along with pKK1,6FucT[8] were used to cotransform XL1Blue-MRF′ cells. Supplementation with GroEL/GroES strongly increased the correct folding of the transferase. Thus, the activity of the soluble fucosyltransferase per liter of culture (6.0 U L^{-1}) was almost twice as high as that found when pKK1,6FucT was expressed alone (Table 1). The effect of overexpression of the chaperonins GroEL/GroES per unit of biomass is even higher than that calculated per liter of culture since the final optical density reached by the culture in the presence of pAG was about 30% lower than that reached in its absence (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 in high purity, but the enzyme was precipitated and inactive after elution from the column. In view of these results, we decided to sub-clone α-1,6-FucT in the pRSET plasmid in order to tag the fucosyltransferase with a polyhistidine tail at the N terminus. The resulting pRSETα1,6FucT plasmid was transformed into BL21(DE3) pLys cells and cotransformed, along with the pAG plasmid, in BL21(DE3) cells. The expression of α-1,6-FucT in BL21(DE3) pLys cells was analyzed after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) at 30 °C. The activity found for the soluble fucosyltransferase (1.3 U L^{-1}, Table 1) was lower than in the system pKK1,6FucT/XL1Blue-MRF′. However, analysis of the expression by SDS-PAGE (Figure 1) showed that the total amount of recombinant enzyme expressed (in soluble form and as IBs) was much higher than in the previous expression system. In the

Table 1. *Activity of soluble α-1,6-FucT in the presence or absence of the GroEL/GroES chaperonin system.*

FucT/Chaperone	Induction T [°C]	Final O.D.	Activity [U L^{-1}][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, which contained 12 mM Hepes (pH 7.7), 50 mM KCl, 6.5 mM $MgCl_2$, 0.7 mM PEP, 0.2 mM NADH, 6 U pyruvate kinase, 13 mU lactate dehydrogenase, 100 μM chitobiose, and 52 μM GDP-Fucose. The assay was initiated by addition of 15 μl CFE and the decrease in the absorbance at 340 nm was monitored. Hepes = *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, PEP = phosphoenolpyruvic acid, NADH = nicotinamide adenine dinucleotide (reduced form).

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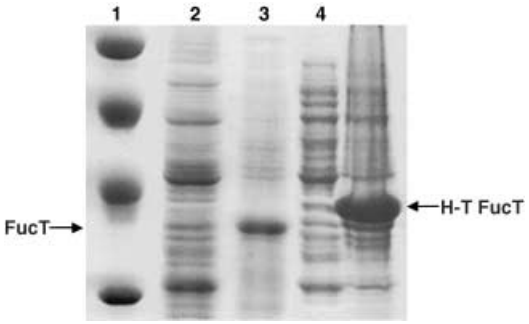


Figure 1. SDS-PAGE analysis[13] of pKK1,6FucT and pRSET1,6FucT expression. Lane 1: molecular weight markers (rabbit muscle phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; chicken egg white ovalbumin, 45 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa); lane 2: soluble fraction of XL1-Blue MRF′ 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. H-T FucT = histidine-tagged α-1,6-FucT.

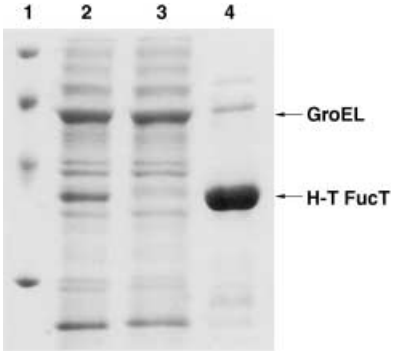


Figure 2. SDS-PAGE for the immobilization/purification of H-T FucT. Lane 1: molecular weight markers as in Figure 1; lane 2: cell free extract (CFE) of BL21(DE3) cells harbouring the pRSET1,6FucT plasmid; lane 3: CFE after passing through an Ni^{2+}-IDA-agarose column; lane 4: Ni^{2+}-IDA-agarose gel after His-tagged FucT immobilization.

case of the BL21(DE3) cells, the increased level of intracellular chaperonins resulted in a significant increase of activity of the soluble α-1,6-FucT (2.3 U L^{-1}, Table 1). Despite the effect of the molecular chaperones, the major portion of the recombinant enzyme remained insoluble. Our next approach to optimize the yield of properly folded α-1,6-FucT was to reduce the growth temperature of the culture to 18 °C. It is well documented that by lowering the cultivation temperature protein aggregation is reduced.[12] In our case, when α-1,6-FucT was expressed at 18 °C in the absence of the pAG plasmid the production of soluble recombinant enzyme was not improved but decreased to 0.8 U L^{-1}, although the cell growth rate was also reduced (Table 1). When GroEL/GroES were coexpressed with the fucosyltransferase at 18 °C the activity of the soluble α-1,6-FucT was increased to 8.0 U L^{-1} (Table 1). This 10-fold increment was much higher than that induced by the same chaperonins at 30 °C, either when 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 we used agarose gels with high concentrations of Ni^{2+} (20–35 μmol mL^{-1} gel) immobilized through IDA ligands. A crude preparation of H-T FucT was directly loaded onto the Ni^{2+}-IDA-agarose column. Figure 2 shows that the H-T FucT was retained by the Ni^{2+}-IDA-agarose in a very selective way (Figure 2, lane 4). The activity of the immobilized α-1,6-FucT was 180–217 mU per gram of gel, with a specific activity of 75.6 mU per milligram of protein. The stability of the immobilized FucT was measured at 4 °C and compared with that of the purified and crude enzyme (Figure 3). The immobilized enzyme was significantly stabilized, with a half-life of 15 days.

Finally, the synthetic utility of the immobilized enzyme was tested in the fucosylation of chitobiose **2** (Scheme 1). The use of

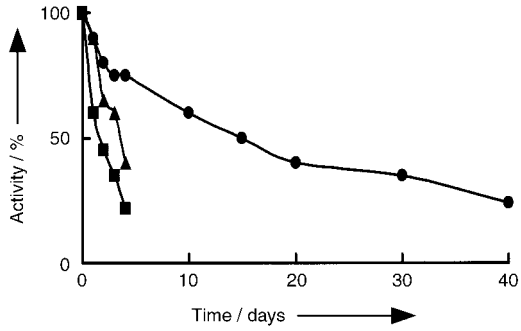
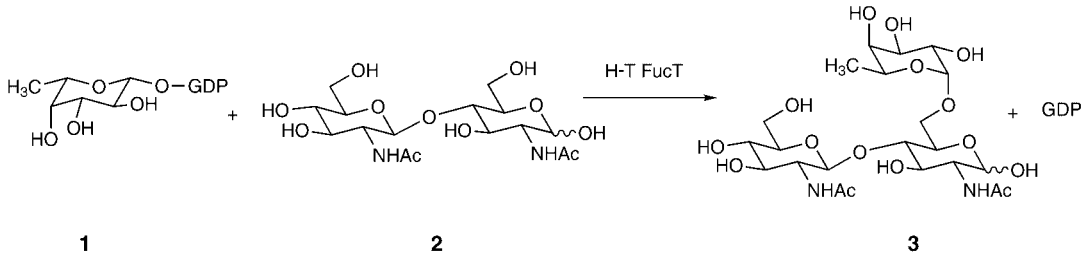


Figure 3. Stability at 4 °C of the immobilized H-T FucT (●), purified H-T FucT (▲), and CFE of BL21(DE3) cells harbouring the pRSET1,6FucT plasmid (■). Immobilized fucosyltransferase was incubated in phosphate buffer (10 mM, pH 7.4) at 4 °C without stirring. At various times, aliquots were taken and assayed for activity. The spectophotometric activity assay for the immobilized FucT was performed whilst stirring the sample. The effect of the Ni^{2+}-IDA-agarose gel on the activity test was evaluated in an assay where the FucT was omited, which showed that the presence of the matrix does not affect the assay.



*Scheme 1. Fucosylation of N,N′-diacetylchitobiose (**2**) using GDP-Fucose (**1**) as the fucose donor, catalyzed by immobilized H-T FucT. GDP-Fucose (**1**; 14.0 μm, 6.0 mg) was incubated with **2** (4.7 μm, 3.0 mg) and Mn^{2+} (10 mM) in the presence of the immobilized enzyme (0.7 g, 0.2 U g^{-1} gel) at 25 °C for 6 h.*

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the immobilized enzyme has several advantages over the use of the soluble one; it allows shorter reaction times because of the higher concentration of the enzyme, the final yield (98%) is higher, and the work-up is much easier because the biocatalyst can be removed by simple filtration.

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[1] a) M. M. Palcic, *Curr. Opin. Biotechnol.* **1999**, *10*, 616; b) K. M. Koeller, C.-H. Wong *Glycobiology* **2000**, *10*, 1157.
[2] a) Z. Ge, N. W. C. Chan, M. M. Palcic, D. E. Taylor, *J. Biol. Chem.* **1997**, *272*, 21357; b) O. Blixt, I. van Die, T. Norberg, D. H. van de Eijnden, *Glycobiology* **1999**, *9*, 1061; c) M. Izumi, G.-J. Shen, S. Wacowich-Sgarbi, T. Nakatani, O. Plettenburg, C.-H. Wong, *J. Am. Chem. Soc.* **2001**, *123*, 10909.
[3] A. Mogk, M. P. Mayer, E. Deuerling, *ChemBioChem*. **2002**, *3*, 807.
[4] A. R. Clarke, *Curr. Opin. Struct. Biol.* **1996**, *6*, 43.
[5] J. S. Weissman, C. M. Hohl, O. Kovalenko, Y. Kashi, S. Chen, K. Braig, H. R. Saibil, W. A. Fenton, A. L Horwich, *Cell* **1995**, *83*, 577.
[6] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, *Nature* **1975**, *258*, 598.
[7] R. R. Beitle, M. M. Ataai in *Immobilization of Enzymes and Cells* (Ed.: G. F. Bickerstaff), Humana Press, NJ, **1996**, pp. 339–343.
[8] A. Bastida, A. Fernández-Mayoralas, R. Gómez Arrayás, F. Iradier, J. C. Carretero, E. García-Junceda, *Chem. Eur. J.* **2001**, *7*, 2390.
[9] A. Bastida, A. Fernández-Mayoralas, E. García-Junceda, *Bioorg. Med. Chem*. **2002**, *10*, 737.
[10] J. Pérez-Pérez, C. Martínez-Caja, J. L. Barbero, J. Gutierrez, *Biochem. Biophys. Res. Commun.* **1995**, *210*, 524.
[11] J. Kamińska, J. Dzięciol, J. Kościelak, *Glycoconjugate J.* **1999**, *16*, 719.
[12] F. Baneyx in *Manual of Industrial Microbiology and Biotechnology* 2nd ed. (Eds: J. E Davies, A. L. Demain, G. Cohen, C. L. Hershberger, L. J. Forney, I. B. Holland, W.-S. Hu, J.-H. D. Wu, D. H. Sherman, R. C. Wilson), American Society for Microbiology, Washington D. C., **1999**, pp. 551, and references cited therein.
[13] U. K. Laemmli, *Nature* **1970**, *227*, 680.

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Selective Protein Degradation by Ligand-Targeted Enzymes: Towards the Creation of Catalytic Antagonists

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The selective degradation of proteins by proteinases is an integral regulatory process that supplements and complements the regulation of expression-controlled protein concentrations. Recently several regulatory proteinase systems have been implicated in functions as diverse as the pathogenesis of Alzheimer's disease[1] or arteriosclerosis[2] through to the facilitation of synaptic processes in the brain.[3] Proteinase-activated receptors constitute a family of G-protein-coupled receptors that are uniquely activated, or indeed deactivated, through partial degradation by extracellular serine proteinases.[4, 5]

The likelihood of the existence of many more unknown regulatory proteinases has highlighted the need for probes that will explore therapeutic potential.[6] Although the selective destruction of proteins by proteinases is a widespread process in nature, the design of de novo degradative systems that can uniquely accomplish elimination or control of the function of a preselected specific target protein has provided a formidable challenge. Pioneering work by Wilchek and co-workers[7] in 1990 explored the possibility of avidin–biotin induced affinity cleavage of streptavidin protein targets by proteinases bearing biotin as a targeting ligand. However, this work showed that indiscriminately biotinylated proteolytic enzymes were not only unable to cleave the avidin protein target but were even less

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