Isotope Labeling of Human Blood Group B Galactosyltransferase for NMR Spectroscopy

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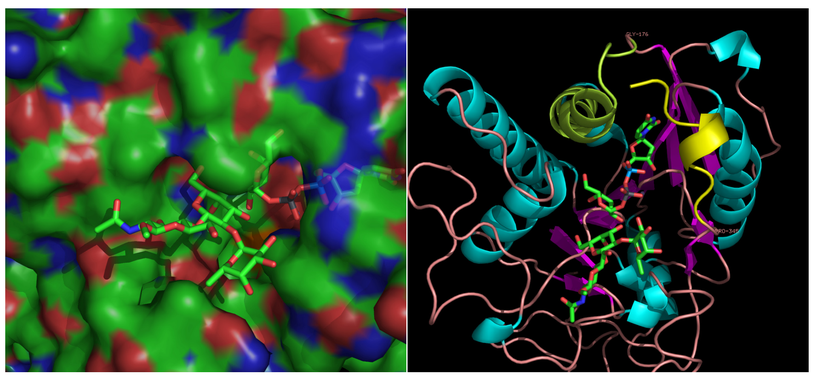
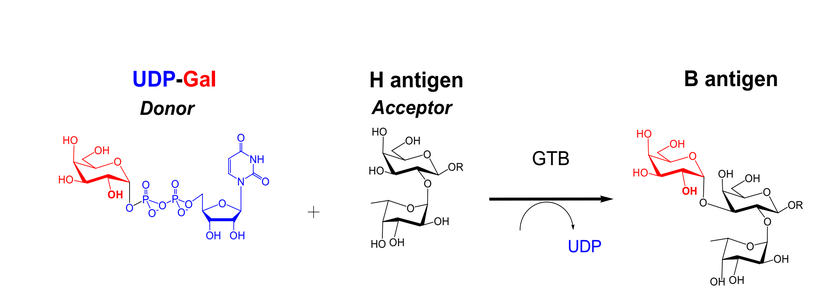
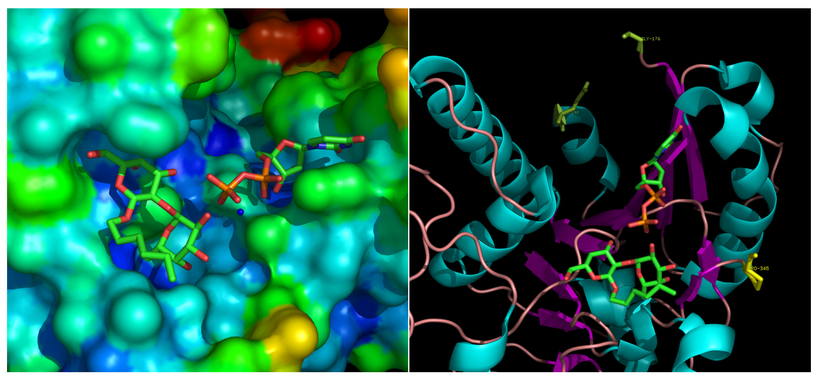
Human Blood Group B Galactosyltransferase (GTB)







GTB is a retaining glycosyltransferase that transfers galactose from UDP-Gal to acceptor saccharides (center). The crystal structure (right panel, [2]) does not reveal the position of the galactose residue and is missing a loop (residues 177-196) and the C-terminal end (residues 346-354). A homology model (left panel, [5]) shows that UDP-Gal should be completely buried in the donor binding pocket.

Open Questions

- **What is the role of the C-terminal loop for the catalytic activity of GTB? How does the catalytic residue Glu303 interact with the donor substrate?**
- **In order to address these questions by NMR, uniformly or selectively $^{13}C,^{2}H,^{15}N$- (CDN)-isotope labeled GTB has to be used.**
- **Strategies for selective labeling of important amino acids in the catalytic site and in the loop are required.**
- **The labeling techniques have to yield high expression rates of GTB to minimize the costs.**

New Achievments

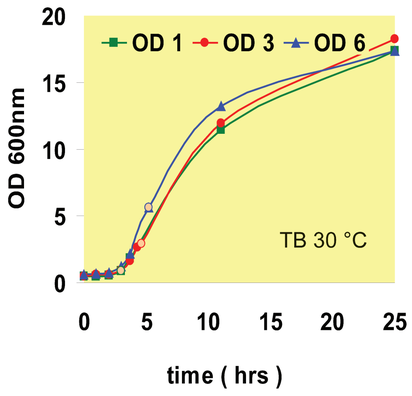
Here we describe for the first time:

- **Expression of uniformly $^{2}H,^{15}N$-labeled GTB (DN-GTB) in high yield with a low cost labeling technique [3].**
- **Expression of selectively $^{1}H,^{15}N$-Asn and $^{1}H,^{15}N$-Met- uniformly ^{2}H-labeled GTB (AM-GTB).**
- **Expression of uniformly ^{15}N-labeled GTB (N-GTB) in high yield.**
- **700 MHz NMR (cryo probe): $^{1}H,^{15}N$ HSQC and $^{1}H,^{15}N$ TROSY spectra of these GTB-samples.**

Optimization of GTB Expression in *E.coli*

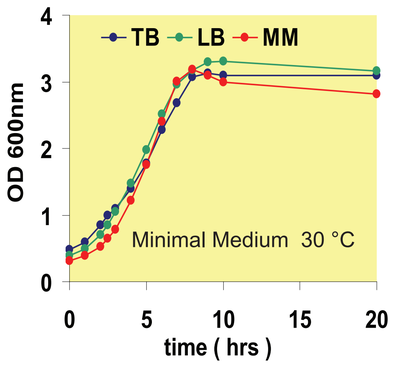
The GTB clone produces in TB full medium as well as in minimal medium 4 mg GTB / 1 g pellet. With standard protocolls for protein expression in minimal medium about 30% of the cell mass, compared to that in full medium, are produced, resulting in a lower yield of recombinant protein. To get high yields of protein a optimized clone as well as optimized conditions for protein expression in full medium are necessary to get the same yield of protein in in minimal medium as in full medium.

Induction



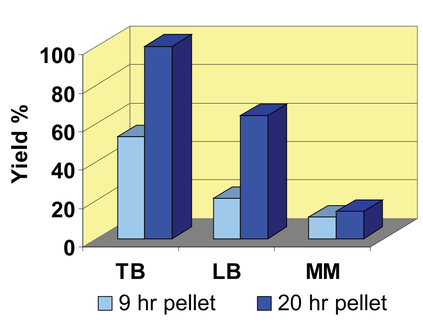
- The expression on rich medium should be optimized.
- Expression should not depend on OD of induction.

Starter culture

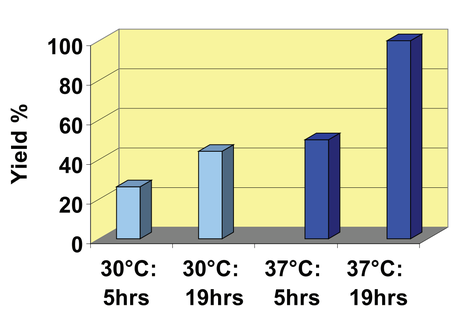


- The yield of recombinant protein in minimal medium depends on the starter culture used and on the optimized supplements to the minimal medium.

Yield in minimal medium

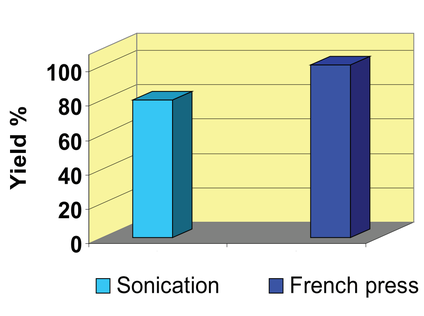


Dependence on temperature



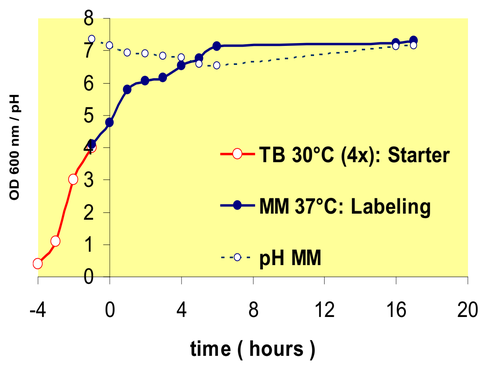
- The temperature for expression in minimal medium can be totally different from the temperature in full medium.
- The time of expression has to be checked carefully. Some clones produce more than 60% of recombinant protein during the stationary phase.

Yield after lysis



- French Press lyses gives about 20% more yield of protein than ultrasonication.

Labeling Strategy

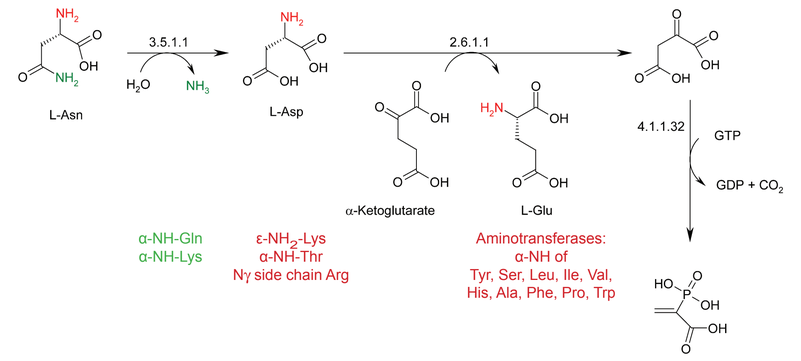


The labeling technique after Marley et al. [3]:

- High yields of labeled protein, significant reduction in costs for labeled compounds, isotopic incorporation >90%.
- Cell mass is generated in unlabeled rich media allowing rapid growth to high cell densities (but cells have to be still in the log-phase!).
- The cell pellet from unlabeled rich medium is used to inoculate isotopically defined labeled minimal medium a higher cell densities, optimized for maximal protein expression.
- The labeling technique can be adapted to all uniform CDN-isotope labeling. No adaption of bacteria to D2O is necessary. For the production of DN-GTB the pellet from 500 ml TB medium at an OD of 4 at 30°C is used to inoculate 500 ml DN-minimal medium (factor 4x).
- The concentration of the antibiotic is doubled to increase the selection pressure in D2O.
- Expression in minimal medium is performed at 37°C, after one hour growing the bacteria are induced. Seven hours after induction the temperature is set to 30°C and protein expression is stopped after 16 hours.

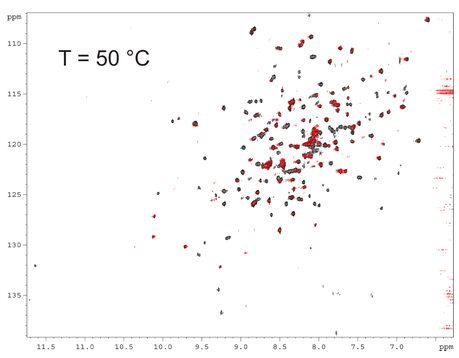
DN-minimal medium composition	final conc.	important
KD_2PO_4/Na_2DPO_4	50 mM	
NaCl	0.05%	
$^{15}ND_4Cl$	0.10%	
D7-glucose	0.40%	
D8-glycerol	0.40%	++
thiamin	20 µg/ml	+
$MgSO_4$	4 mM	+++
$MgCl_2$	1 mM	+
$CaCl_2$	0.1 mM	
$FeSO_4$, $CoCl_2$	1 µM	+
$ZnCl_2$	0.2 mM	+
vitamin solution BME, Sigma	1x	++
DN-uniform labeled full medium	10%	++
Amp	200 µg/ml	+
pH	7.2 - 7.5	+
temperature	37°C	+++
time of expression	18 hrs	+++
air supply		++

Scrambling of Labels

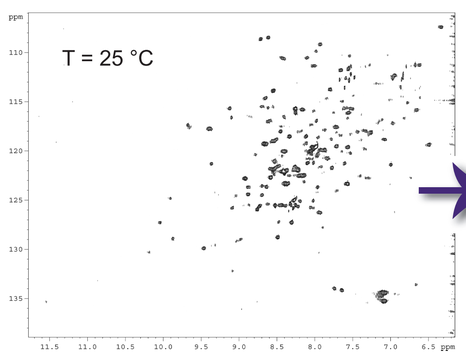


For the production of **AM-GTB** 250 mg $^{15}N_2$-Asn and 125 mg ^{15}N-Met were added to 500 ml minimal medium. Unfortunately, the bacteria preferred the $^{15}N_2$-Asn instead of glucose as general carbon source, and therefore the ^{15}N-label was distributed over a wide variety of other amino acids (scrambling) [4]. For selective labeling, minimal media with all individual amino acids weighed in separately has to be used. The yield in medium that contains all amino acids is similar to the yield in full medium, but the cost of labeling is extremely high.

NMR Experiments



Overlay of $^{1}H,^{15}N$ TROSY spectra (50°C) at 700 MHz of 800 µM **DN-GTB** (black) and 600 µM **N-GTB** (red) in 25 mM BisTris pH 6.5.*



$^{1}H,^{15}N$ TROSY spectrum (25°C) at 700 MHz of 800 µM **DN-GTB** in 25 mM BisTris pH 6.5.*

* 25 mM BisTris, pH 6.5 (RT), 50 mM NaCl, 10 mM MgCl2, 5 mM 2ME

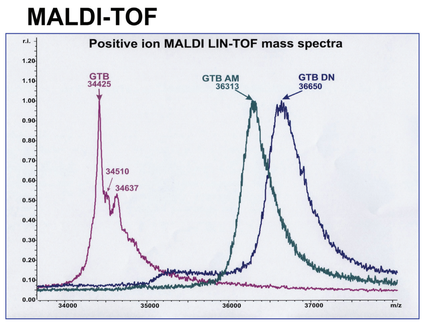
Yield and costs of labeled GTB				
Medium	TB	N-MM	DN-MM	Standard DN-labeling
Volume	1000 ml	1000 ml	1000 ml	1000 ml
€ / L	40 €	60 €	2,800 €	2,800 €
mg / L	100 mg	100 mg	120 mg	28 mg
€ / mg	0.40 €	0.60 €	23 €	100 €

For the analysis of ligand binding to GTB using chemical shift perturbation methods it is mandatory to uniformly label the enzyme with ^{2}H and ^{15}N. This yields well resolved $^{1}H,^{15}N$-TROSY spectra, even at moderate temperatures (25°C, right panel DN-GTB (black)).

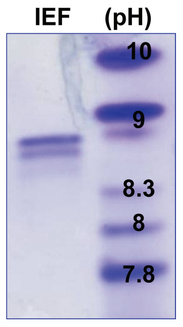
Protein Analysis

The incorporation level of DN is >90% as determined with MALDI-TOF. The difference in MW between the **DN** and **AM**-labeled **GTB** is smaller than calculated, indicating a higher incorporation of $^{1}H,^{15}N$-label (scrambling). Labeled and unlabeled GTB show two isoforms in the isoelectrofocussing gel.

MALDI-TOF



IEF (pH)



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Acknowledgments:

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