Isotope labeling of human blood group B galactosyltransferase and human very low-density lipoprotein receptor V3 for NMR experiments

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Open questions

GTB is a retaining glycosyltransferase that transfers galactose from UDP-Gal to acceptor saccharides. The crystal structure [1] does not reveal the position of the galactose residue and is missing a l oop (residues 177-196) and the C-terminal end (residues 346-354). A homology model [2] shows that UDP-Gal should be completely buried in the donor binding pocket.

- **What is the role of the C-terminal loop and the C-term end for the catalytic activity of GTB?**
- **How is the mechanism of the catalytic reaction?**

V3: Human rhinoviruses are a major cause of the common cold. Minor group viruses, such as HRV2, use members of the low- density lipoprotein receptor (LDLR) family for cell entry. The ligand binding domain of these receptors consists of various numbers of cysteine-rich modules and the crystal structure of HRV2 in complex with two such repeats (V23) was determined at 3.6 Å [3]. For our studies we have used the V3 module of the very low density lipoprotein receptor (VLDLR). Mapping of the binding epitope of a single module by saturation transfer difference NMR is possible [4] but requires prior assignment of the binding module which necessitates its labeling.

- **How is the structure of different single modules in solution / in complex with rhinoviruses?**
- **How determines the binding epitope differences in affinity?**

New achievments

- In order to address these questions by NMR, uniformly or selectively $^{13}C,^{2}H,^{15}N$- (CDN)-isotope labeled proteins have to be used.
- Strategies for selective labeling of important amino acids are required.
- The labeling techniques have to yield high expression rates to minimize the costs.

Here we describe for the first time:

- Expression of uniformly $^{2}H,^{15}N$-labeled GTB in high yield with a low cost labeling technique [3].
- 700 MHz NMR (cryo probe): transfer NOESY spectra of $^{2}H,^{15}N$-GTB [5].
- Optimization of the expression of uniformly ^{15}N-labeled V3 receptor fragment in high yield.

Principle aspects about labeling:

- For each clone optimization of medium and expression conditions usually result in higher yield of protein.
- The labeling conditions for two truncated forms of the GTB, suffering only 5 or 10 amino acids from the N-term end were totaly different to those shown here (e.g. growth time, concentration factor, temperature).
- VLDLR V3 receptor fragment is expressed as a fusion protein with MBP. After cleavage only 15 % of the protein is V3. Without optimization of expression isotopic labeling would be too expensive.
- After new transformation and optimization of all mediums the yield of labeled V3 increased about 6-fold.

Optimization of protein expression in E.coli

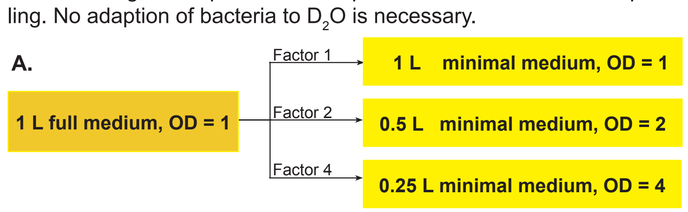
In general clones produce the same amount of recombinant protein per 1 g of wet pellet in full medium as well as in minimal medium. With standard protocols for protein expression in minimal medium only about 30% of the cell mass is obtained compared to full medium.

- To obtain high yields of protein the conditions and especially the composition of full medium as well as of minimal medium have to be optimized before isotopic labeling.
- To get nearly the same yield of protein in minimal medium as in full medium, the labeling technique of Marley et al. [6] is extremely useful. Amounts of cell mass are produced in full medium and are used to inoculate minimal medium at high cell densities.
- The yield of GTB (MW 34 kDa) in rich medium (TB) is 100 mg/L. After optimizing the composition of the minimal medium (MM) we obtained 120 mg/L $^{2}H,^{15}N$-GTB.
- The yield of V3 receptor fragment (MW 7.3 kDa) in LB medium was 70 mg/L for the fusion protein MBP-V3 (49 kDa). After optimization of the composition of the rich medium (V8) we obtained 1 g/L MBP-V3 and for minimal medium (MM3) the yield of ^{15}N-MBP-V3 was 700 mg/L. After cleavage and refolding the yield of V3 was 100 mg/L respectively 40 mg/L.

Labeling Strategy

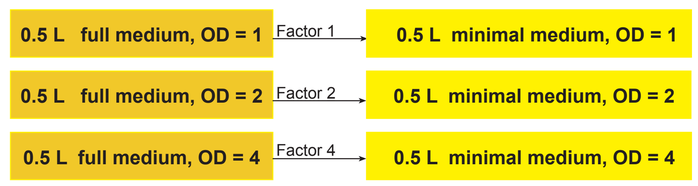
A: The labeling technique after Marley et al. [3]:

• 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 D_2O is necessary.



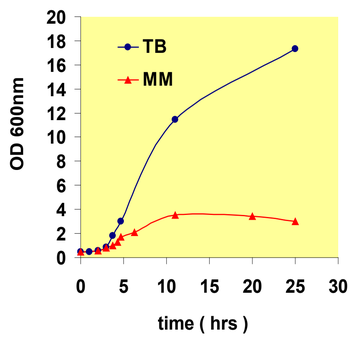
B: Modified labeling protocol

• When the concentration factor after Marley et al. is 4 and a high concentration of the labeled protein is needed, it can be difficult to manage the high volumes of rich medium with an OD of 1 to produce the cell mass needed for inoculation in minimal medium.
• If the clone accepts the induction at OD 4 without any loss in protein yield it is possible to inoculate the same volume of minimal medium with the bacterial pellet at an OD of 4 of the same volume of rich medium. This corresponds to a concentration factor of 4 after Marley et al..



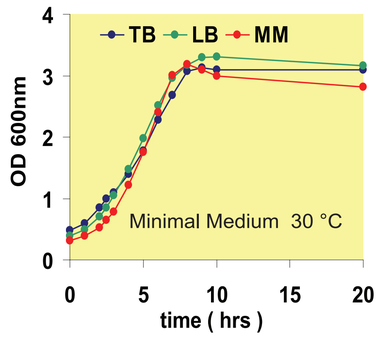
Principle techniques for optimization of the expression of isotopically labeled proteins in E.coli

Growth curve GTB



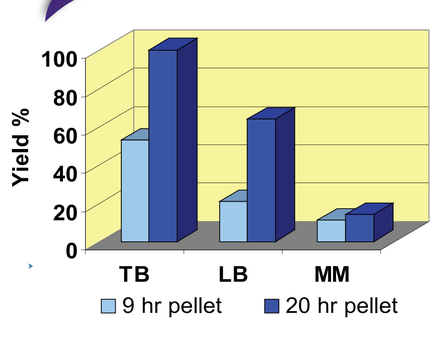
- The weight of the wet pellet from full medium is 20 g/L (GTB: 4 mg/1 g), whereas in minimal medium only 9 g/L wet pellet can be collected.
- The expression time in minimal medium is shorter than in TB medium.

Starter culture is important

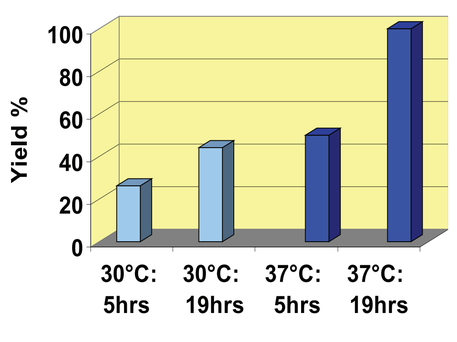


- The growth curves in minimal medium do not show any difference although different starter cultures (TB, LB, MM) were used.
- Even when the composition of the minimal medium is optimized, the growth curve may not significant differ from growth in not optimized medium.
- The yield of recombinant protein in minimal medium depends strongly on the starter culture used.

Yield in minimal medium

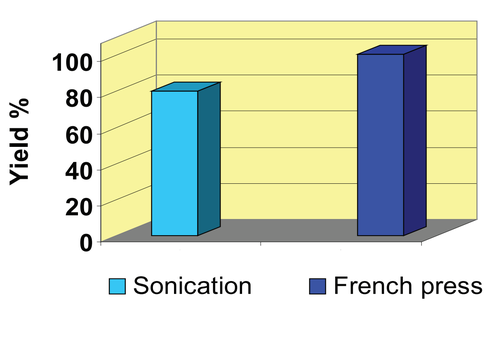


Temperature and expression time



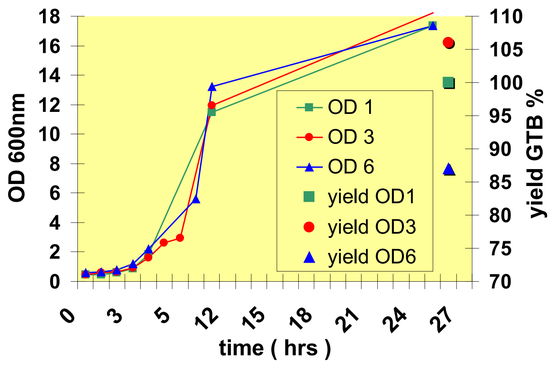
- The temperature for expression in minimal medium can be totally different from the temperature used for expression in full medium.
- The time of expression has to be checked carefully. Some clones produce more than 60% of recombinant protein during the stationary phase.

Yield after lysis



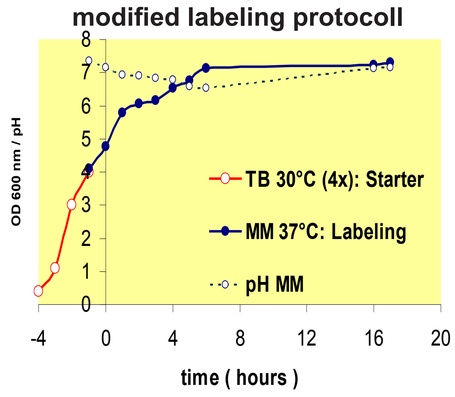
- French Press lyses gives about 20% more yield of protein than ultrasonication. GTB is extremely sensitive to sonication resulting in lower yield.

GTB yield after high OD induction



- The expression on rich medium should be optimized.
- Expression should not depend on OD of induction. The yield of GTB did not decrease until induction at OD 4.

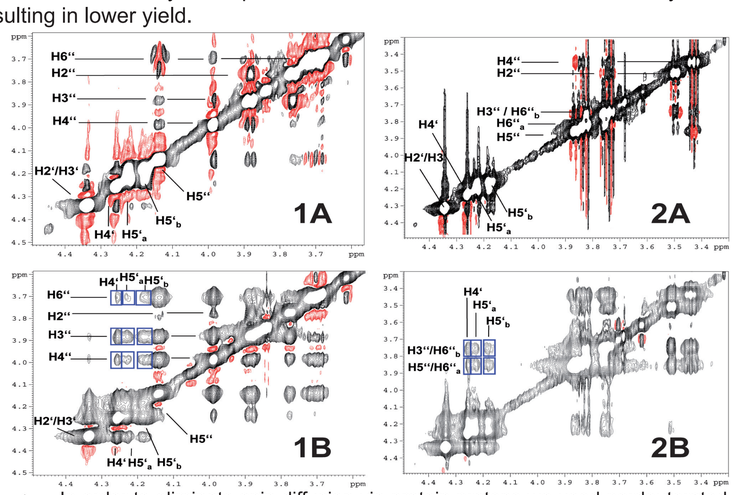
$^{2}H,^{15}N$-labeling of GTB after modified labeling protocoll



• For the production of DN-GTB the pellet from 500 ml TB medium at an OD of 4 is used to inoculate 500 ml DN-minimal medium (concentration factor 4x).
• 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		++

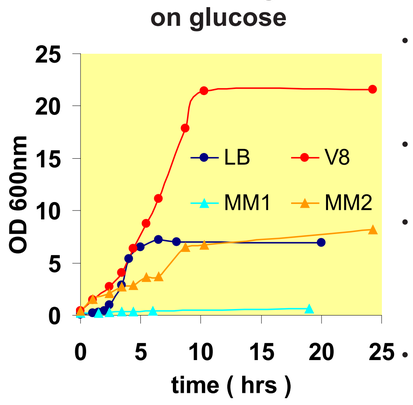
• Most important (+) for a high yield of protein was Mg^{2+}, as well as B-vitamins and 10% labeled full medium.
• 200 g/ml Amp were used for high selection pressure in D_2O.
• The pH-value should never decrease below pH 6,5.



- In order to eliminate spin diffusion via protein protons we used perdeuterated $^{2}H,^{15}N$-GTB for the transferred NOE experiments. From MALDI-TOF spectra we estimated the extent of deuteration to be larger than 95%.
- ^{1}H-NOESY spectra of a complex of $^{2}H,^{15}N$-GTB with UDP-Gal (1) and UDP-Glc (2) in the absence (A) and in the presence (B) of Mg^{2+} ions* at 700 MHz. The protein : ligand ratio is 1:2.
- The NOESY spectra for the complex with Mg^{2+} ions unambiguously show negative NOE cross peaks [5]. NOEs across the pyrophosphate bridge (blue squares) are only observed for the bound states of UDP-Gal and UDP-Glc.

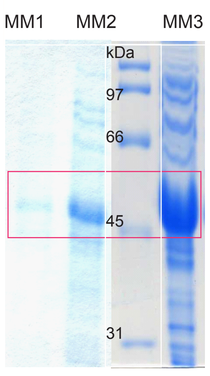
Growth curves MBP-V3

TB1 clone: no growth on glucose



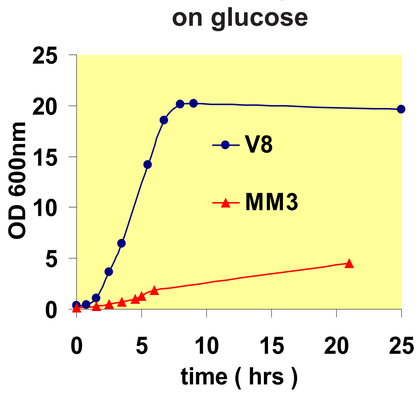
- The growth curve of the V3 clone in optimized V8 full medium results in significant higher cell mass than in LB medium.
- Expression in minimal medium with glucose (or/and glycerol) as solely C-source (MM1) was not possible in TB1 cells.
- Only in labeled full medium (MM2) containing labeled amino acids significant amount of V3 was expressed.
- The concentration factor after Marley et al. was only 1 for inoculation in MM2. This means that no cost reduction for the isotopic labeling is possible with the clone in TB1 cells.
- The SDS gels shows the expression of MBP-V3 in minimal medium with and without glucose in TB1 and BL21 cells.

V3 SDS-PAGE



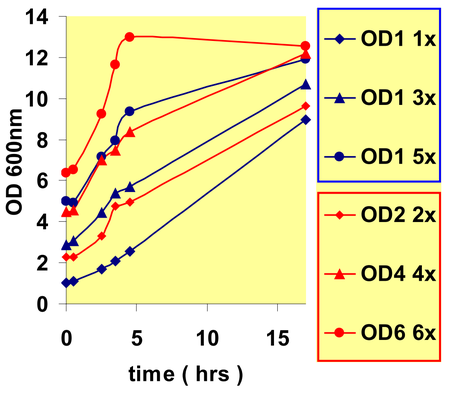
MM1: glucose / TB1
MM2: amino acids / TB1
MM3: glucose / BL21

BL21 clone grows on glucose



- After a V8-plasmid transformation in BL21 cells the growth curve in V8 full medium was similar to that of TB1 cells in V8.
- With the modified labeling protocol a higher cell mass and higher yield of MBP-V3 were obtained.
- The comparision of the concentration factors showed that higher factors were obtained with the modified protocol.
- With the new clone and the modified labeling protocol the yield of V3 and the costs for isotopic labeling have been significantly reduced.
- The concentration factor increased from 1x in TB1 to 6x in BL21 cells.

MBP-V3 growth curve in MM3: Marley vs. modified protocol



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* NMR-buffer: 25 mM BisTris, pH 6.5 (RT), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM 2ME