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On the efficient bio-incorporation of 5-hydroxy-tryptophan in recombinant proteins expressed in *Escherichia coli* with T7 RNA polymerase-based vectors

Wellington P. Oliveira-Souza [a], Fellipe Bronze [a], Jaap Broos [b], Marcelo F.M. Marcondes [a, b, *], Vitor Oliveira [a]

[a] Department of Biophysics, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Pedro de Toledo, 669–7° Andar, São Paulo, Brazil
[b] Groningen Biomolecular Sciences and Biotechnology Institute, Biophysical Chemistry, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

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ABSTRACT

Biosynthetic incorporation of non-canonic amino acids is an attractive strategy to introduce new properties in recombinant proteins. Trp analogs can be incorporated in recombinant proteins replacing regular Trp during protein translation into a Trp-auxotrophic cell host. This straightforward method however, is limited to few analogs recognized and accepted by the cellular protein production machinery. 5-hydroxy-tryptophan (5OH-Trp) can be bio-incorporated using *E. coli* as expression host however; we have experienced very low incorporation yields - amount of protein containing regular Trp/amount of protein containing the Trp analog – during expressions of 5OH-Trp labeled proteins. Furthermore, this low incorporation yield were verified especially when the widely-used vectors based on the T7 RNA polymerase were used. Testing different 5OH-Trp incorporation protocols we verified that in these T7-based systems, the production of the T7 RNA polymerase is driven by the same elements – lac promoter/IPTG – as the target protein. Consequently, the bio-incorporation of the 5OH-Trp residues also occurs in this crucial enzyme, but, the produced T7 RNA polymerase labeled with 5OH-Trp is inactive or much less active. In the present work, we describe an efficient method to overcome this mentioned problem and bio-incorporate 5OH-Trp in proteins expressed in *E. coli*., using vectors based on the T7 RNA polymerase-T7 promoter. The two-step induction protocol here described showed incorporation efficiencies of 5OH-Trp higher than 90%.

1. Introduction

Tryptophan residues can be used as spectroscopic probes to explore protein structure. Taking advantage of Trp fluorescence properties, spectroscopic data can be used to study protein-protein interactions, protein-ligand binding, protein folding and etc. [1–5]. Incorporation of tryptophan analogs into a protein may facilitate and expand these possibilities: 5-hydroxy-L-tryptophan (5OH-Trp), for instance, absorbs at 310–320 nm, allowing its specific excitation in a Trp background [6–8]. 5OH-Trp as well as other Trp analogs can be incorporated in recombinant proteins replacing regular Trp during protein translation into a Trp-auxotrophic cell host. Usually, the auxotrophic strain is grown in minimal media in the presence of "regular" Trp till the culture reaches the exponential phase. Cells are collected and washed to remove Trp and then resuspended in minimal media containing the exogenous Trp analog, and protein expression is induced [9,10]. Tryptophan isosteres analogs like fluor-Trp can be incorporated in the place of regular Trp yielding target protein efficiently labeled with fluor-Trp analog. However, most studies in which another Trp analog than fluoro-trp was used, including 5OH-Trp, lower labeling efficiencies were observed. Furthermore, in *E. coli* expressions, for instance, replacement of Trp by its bulkier analogs like Bromo-Trp and Nitro-Trp does not lead to any measurable amount of Trp analog labeled protein [10]. Many

Abbreviations: α-SN, alfa-synuclein; α-SN^{39} is the general abbreviation used for the protein resultant of the α-SN Tyr39Trp mutant gene expression, and, after identification by HPLC analysis, α-SN^{39}Trp for the protein containing a Trp at position 39 or α-SN^{39}5OH-Trp for the protein containing a 5OH-Trp at this position 39.

* Corresponding author. Universidade Federal de São Paulo (UNIFESP), Department of Biophysics, Rua Pedro de Toledo, 669, 04039-032, Enzymology Laboratory–7th floor, São Paulo, Brazil.
E-mail address: marcelo.marcondes@unifesp.br (M.F.M. Marcondes).

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reasons can be pointed out for these differences, including the specificity of the tRNA-Trp synthase and the position of the Trp residue(s) in the target protein to be replaced. But, the extend protein labeled with the Trp analog is also dependent on the expression system used [11–13]. Most proteins labeled with a Trp analog have been produced using an *E. coli* Trp auxotrophic strain and currently the use of T7 RNA polymerase based system is one of the most popular *E. coli* expression methods. The T7 promoter can also be tightly regulated and numerous T7 promoter based expression vectors are commercially available [14]. This system depends of the production of the T7 RNA polymerase upon introducing inducer and only the produced T7 RNA polymerase can transcribe the gene of the target protein upstream the T7 promoter [15]. *E coli* strains, including Trp auxotroph strains, transformed with the λDE3 become T7 RNA producing strains [16].

In this work, the potential of T7 based expression systems for efficient labeling of proteins with a Trp analog is explored via modifications introduced in the expression protocol. As T7 RNA polymerase has 19 Trp residues in its structure [17] we realized that in a standard Trp analog bio-incorporation protocol, as resumed above, such Trp residues would also be replaced by the analog, therefore, we hypothesized that replacing these 19 residues with a Trp analog, like 5OH-Trp, it can lead in a T7 RNA polymerase not functional (T7 RNA polymerase inactive or less active at least). Our work shows that relative simple modifications can significantly enhance the labeling efficiency to ~90%, together with a high protein yield.

2. Materials and methods

2.1. Plasmid constructions

The pT7-7 plasmid containing the coding sequence for wild α-SN was acquired from Addgene (plasmid # 36046) (Addgene, Cambridge, MA, USA) [18]. Using specific primers the α-SN codon 39, encoding a tyrosine residue was change by PCR to the tryptophan codon yielding α-SN Tyr39Trp. Synthetic oligonucleotides (primers) were also used for α-SN wild type and α-SN Tyr39Trp, subcloning into pET26b (Merck KgaA, Darmstadt, Germany) vector between the *Nde*I and *Xho*I restriction sites.

2.2. E. coli tryptophan auxotrophic strains - CY15077 and W3110 TrpA88

The λDE3 prophage was introduced in E. *coli* Trp auxotrophic strains CY15077 and W3110 TrpA88 using a λDE3 lysogenation kit (Merck KgaA, Darmstadt, Germany).

2.3. Expression of α-SN - pT7-7 or pET26b

LB medium supplemented with ampicillin (100 μg mL^{-1}) (pT7-7 vector) or with kanamycin (50 μg mL^{-1}) (pET26b vector) was used to make an initial overnight pre-culture of one transformed Trp auxotroph *E. coli* colony in a shaker at 37 °C at 160 rpm. M9 minimal medium also supplemented with the appropriate antibiotic marker and containing 1 mM Trp was innoculated (1:50) with the overnight pre-culture and was grown till an $O.D_{600}$ of 0.6–0.8. At this point one of two different protocols was followed:

α-SN wild-type and α-SN^{39} expressions using a regular protocol expression for Trp analog bio-incorporation. In this protocol the cells were washed using centrifugation (20 min, 3200 g) as described [10] and re-suspended in fresh M9 medium to which was added 5OH-Trp 1 mM or Trp 1 mM with or without IPTG 1 mM (or a different concentration as indicated when required in the text). The culturing was continued for 4 h.

α-SN wild-type and α-SN^{39} expressions using a modified two-step induction protocol. In this protocol IPTG 1 mM (or a different concentration as indicated when required in the text) was added when the bacterial growth reached $OD_{600} = 0.6$ and after 30 min the cells were washed as described [10] and re-suspended in M9 medium containing 5OH-Trp or regular Trp (control experiments) with or without IPTG 1 mM (or a different concentration as indicated when required in the text). The culturing was continued for 4 h.

2.4. Purification of α-SN

Pelleted cells were suspended in 4 ml of binding buffer (10 mM Tris, pH 7.4, 10 mM EDTA, 10 mM E-64 and 1 mM PMSF). Cell lysis was performed in a French Press (Thermo scientific, Waltham, MA, USA). After that the lysate was centrifuged 10 min at 27.000 g and the supernatant was collected.

α-SN was purified by ion exchange chromatography using a Resource Q resin (GE Healthcare, Chicago, IL, USA). Purification was accomplished by binding the protein to the resin using binding buffer (10 mM Tris, pH 7.4), followed by wash step using 5 ml binding buffer. α-SN was eluted using a linear gradient of 0–1 M NaCl in 10 mM Tris, pH 7.4.

Buffer salts were exchanged using a Desalting Prep column (GE Healthcare, Chicago, IL, USA) equilibrated and eluted with $(NH4)_2CO_3$ 40 mM pH 7.4.

2.5. High performance liquid chromatography (HPLC)

The resultant purified α-SN^{39} mutant proteins were analyzed by high performance liquid chromatography (HPLC). Analytical reverse phase chromatography on a C4 column (300 Å, 5 μm, 4.6 mm × 150 mm) with a linear gradient elution was used: (A) TFA/H_2O (1: 1000) and (B) TFA/acetonitrile/H_2O (1: 900: 100). Flow rate 2 ml/min. Elution gradient of 10–80% (B) in 40 min. Equipment: Shimadzu LC10Avp fitted with a fluorescence detector RF-10AXL and a diodearray detector SPD-M10Avp (Shimadzu Co., Kyoto, Japan).

3. Results and discussions

The development of a protocol for the efficient biosynthetic incorporation of 5OH-Trp in recombinant proteins using a T7 based expression system, started with choosing a suitable target protein. The intrinsically disordered protein α-synuclein (α-SN) was selected because this protein as well as the α-SN Tyr39Trp single Trp mutant can be easily produced using a T7 promoter based expression vector [19–21]. Furthermore, the intrinsically disordered nature of α-SN [22] makes this protein a reasonable target model-test protein, since it is very likely that position 39 is permissive when labeled with Trp analogs as the 5OH-Trp.

A first assay was made in order to verify our hypothesis that the replacement of Trp residues in the T7 RNA polymerase could be a “bottle of neck”. Briefly, the culture was grown in the presence of regular Trp till the OD = 0,6–0,8, at this point the culture was centrifuged and the bacterial pellet washed twice using the M9 medium without any Trp, after the wash step the pellet was re-suspended in the M9 medium and divided in 2 cultures: **1** - regular Trp was re-introduced and **2** - 5OH-Trp was added. An induction period using 1 mM of IPTG during 4 h at 37 °C was then followed and the final cell cultures were analyzed by SDS PAGE (Fig. 1). By using this “regular protocol” a good α-SN^{39} expression yield was observed in the culture **1** which Trp was re-introduced (Fig. 1 lane 07), but no α-SN^{39} expression was observed in the culture 2 with the 5OH-Trp addition (Fig. 1 lane 08) prior to the induction step.

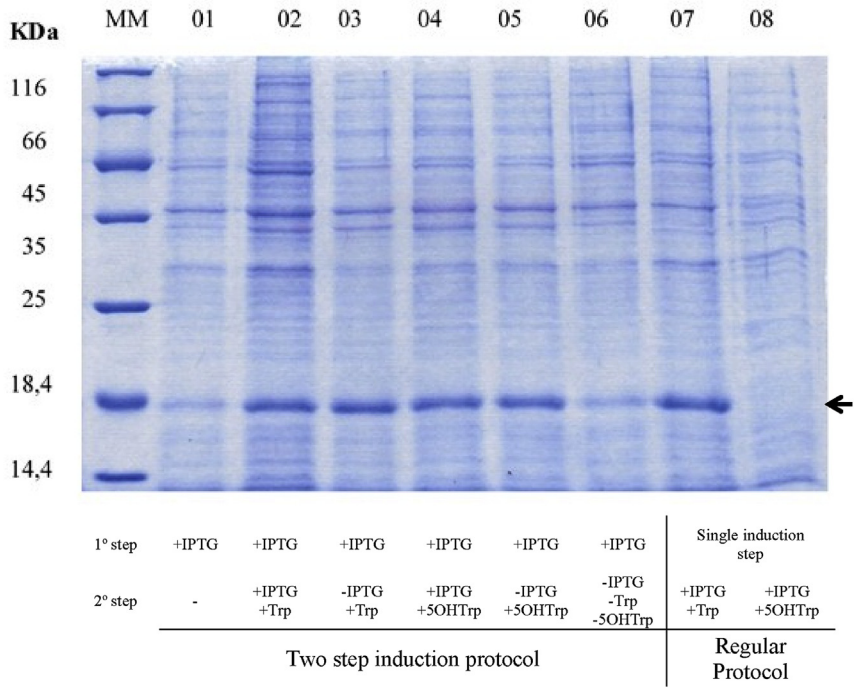

Fig. 1. α-SN^{39} expression in CY15077 λDE3 lisogenic. **Lanes 07 and 08—regular Trp analog bio-incorporation protocol**. Lane 07—re-introduction of regular Trp. Lane 08—change for 5OH-Trp. **Lanes 01 to 06—two step induction protocol**. Lane 01—aliquot took after the initial 30 min induction with 1 mM of IPTG, but before the change for the medium with 5OH-Trp or regular Trp (1° step control). Lane 04—change for the 5OH-Trp and re-addition of 1 mM of IPTG. Lane 05—change for the 5OH-Trp without IPTG. Lane 02—re-introduction of regular Trp and 1 mM of IPTG. Lane 03—re-introduction of regular Trp without IPTG. Lane 06—Second induction period, no addition of Trp or 5OH-Trp and without IPTG (2° step control). Arrow indicates the α-SN bands in the Figure.

As mentioned in the introductory section, many reasons can be pointed out for this no α-SN^{39} expression following the IPTG induction + 5OH-Trp. Among those possibilities is that the produced T7 RNA polymerase in this method maybe be inactive or, less active at least. Therefore, some modifications in the protocol were planned in order to avoid the T7 RNA labeling by the 5OH-Trp. A two-step induction protocol was then applied. In this approach the culture grown in the presence of Trp till the OD = 0.6–0.8, at this point 1 mM of IPTG was added and the culture followed by an incubation period of 30 min that allow the expression of the T7 tRNA polymerase (Fig. 1 Lane 01), and, after this first short induction period the cultures were treated as the regular protocol, a washing step to remove all Trp from the medium followed by the second induction period using 1 mM of IPTG during 4 h but with different conditions: Trp (Lane 02), 5OH-Trp (Lane 04) and a control without inducer and no addition of Trp or 5OH-Trp (Lane 06). This protocol change lead to a good expression level of α-SN^{39}, even when 5OH-Trp was added and with a comparable yield as the achieved with Trp. The amount of α-SN^{39} in the controls (lanes 01 and 06) indicates a low expression in the first induction period that only increased when Trp were re-introduced (lane 02) or 5OH-Trp were added (lane 04).

Fig. 1 also shows the results of another set of samples that were made to verify the "life time" of the T7 polymerase produced in the first induction period, and further check if the T7 RNA polymerase produced in the first 30 min induction was enough to sustain the expression process. For that no IPTG was added in the second induction period: Fig. 1 lane 03 shows the analysis of the sample containing Trp and Fig. 1 Lane 05 the sample containing 5OH-Trp. It is interesting that both testes show similar results.

Taken together these results corroborate the importance of Trp residues on the activity of the T7 polymerase, and shows that the enzyme expressed in the first step of induction are sufficient to produce high levels of α-*SN*^{39} labeled with Trp (α-*SN*^{39}Trp) but especially also with the 5OH-Trp (α-SN^{39}5OH-Trp). However, the expression yield of this methodology depends of the T7 polymerase expression for a short time in the regular Trp containing medium, and, it occurs together with the expression of the target protein produced concomitantly (Fig. 1 lane 1) what can lead to an undesirable large amount of α-SN^{39}Trp. But, a good expression protocol for protein labeling with Trp analogs should provide also a high incorporation yield (IY), that can be defined as the ratio between the fraction of produced protein containing 5OH-Trp and the fraction of produced protein containing Trp (equation (1)).

$$IY\ (\%) = \left(\frac{[\alpha SN^{39}5OHTrp]}{[\alpha SN^{39}5OHTrp] + [\alpha SN^{39}Trp]}\right) \times 100 \quad (1)$$

Therefore, the α-SN^{39} samples were subsequently analyzed and the incorporation yields determined. Trp labeled and 5OH-Trp labeled α-SN^{39} could be base-line separated by reverse phase HPLC chromatography. The elution was followed by monitoring the absorbance at 220 nm and 310 nm. At 220 nm both α-SN^{39} containing Trp or 5OH-Trp have the same molar extinction coefficient but, as only the 5OH-Trp absorbs at 310 nm this feature was used as control. Fig. 2 represents a typical comparison between chromatograms of a HPLC run.

The integrated area of the chromatograms reveals a low 5OH-Trp incorporation yield of 27% (Table 1), what may result due to the α-SN^{39}Trp production in the first induction step that occurs in the Trp containing medium. Therefore, we initially tested this possibility by interfering in the transcription during the induction step. The ionic strength can influence the T7 RNA polymerase binding to its promoter [23], this characteristic was then used to avoid the target gene transcription during the induction step, in order to, consequently, decrease the "basal" α-SN^{39}Trp. For that ammonium

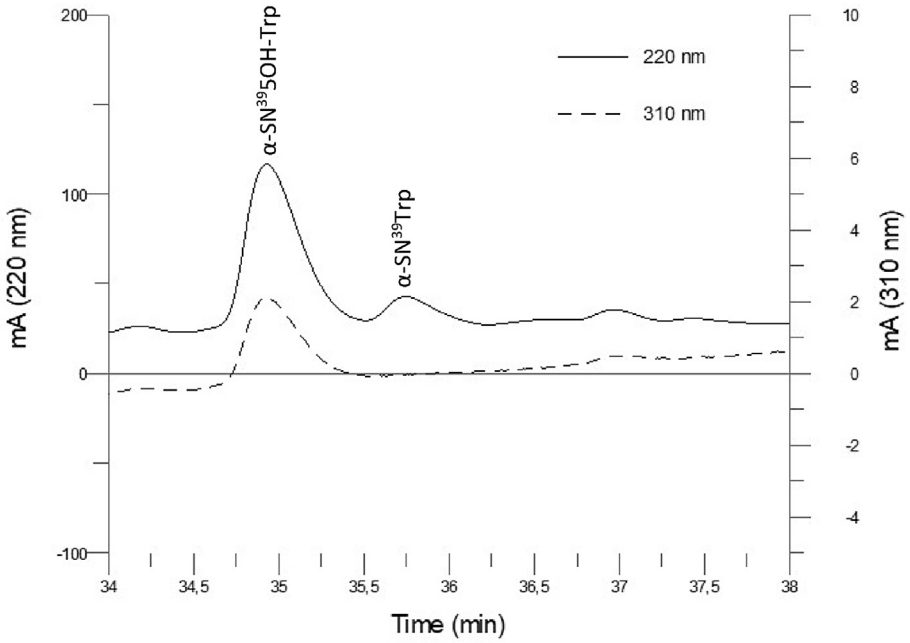

Fig. 2. HPLC analysis of a purified α-SN^{39} sample. Chromatogram showing the separation of the expressed and purified α-SN^{39} containing regular Trp (α-SN^{39}Trp) from the α-SN^{39} containing 5OH-Trp (α-SN^{39}5OH-Trp). mA – milli absorbance unit.

sulfate 400 mM was added together with the inducer in the first induction period and after 30 min the medium was exchanged to a regular (no ammonium sulfate) M9 medium + or−5OH-Trp and expression continued for 4 h. Analysis of the resultant α-SN^{39} purified samples showed that this approach really increased the incorporation yield, from 27 to 89%.

3.1. Transcription regulation effects on incorporation yield

It has been described that a host system with a leaky promoter can result in expression of target protein while the cells are in regular Trp containing medium, lowering the overall percentage of Trp analog labeled protein. Tightly regulated *lac*, *tac* or heat inducible λP_L promoters have been used successfully to yield protein efficiently labeled with the chosen Trp analog [11–13]. Consequently, a in the T7 based system another way to increase the incorporation yield can be made by controlling the transcription of the T7 polymerase and/or the target protein since, it is known that vectors carrying the T7 promoter, when used in rich medium shows an expression even in the absence of inducer, it occurs because the gene of T7 polymerase is downstream of the lacUV5 promoter and in rich medium any trace of lactose can induce the expression of the T7 polymerase, however, a lac operator positioned downstream of the λDE3 lysogens promoter allow for example, a regulation of the T7 polymerase expression by adding glucose into medium [24].

Glucose is the most used carbon source in the expression protocols using minimal medium, and can provide a tightly regulation of the T7 polymerase level by using different concentrations of inducer (IPTG). T7 based vector can be classified in two categories according with the promotor regulation, T7*plain* or T7*lac* promoter. Fig. 3 shows a comparative scheme of the vectors carrying the "plain" T7 promoter and the vectors carrying the T7 lac promoter [24]. It occurs because the T7*lac* promoter vector shows a powerful combination; these vectors contain a lac operator sequence downstream of T7 promoter, and also carry the natural cassette that allows the expression of the lac repressor (lac I). When this type of vector is used the lac repressor can acts in both ways, regulating the expression of the T7 polymerase by acting in the lacUV5 promoter and in the transcription of the target protein via T7 promoter regulation.

This double regulation present in the T7*lac* vector could be explored in our two-step induction protocol; for example, using different concentration of IPTG in the first induction period could be a way to find a good ratio between the expression of the T7 polymerase and the target protein. Ideally searching a good expression of the T7 polymerase, necessary in the second induction step, associated whit a low expression of the α-*SN*^{39}Trp that contribute for increasing the ration between 5OH-Trp labeled/Trp labeled α-SN^{39}. In the second induction step the amount of IPTG should enhance the incorporation yield by increasing the concentration of 5OH-Trp labeled α-SN^{39}.

In order to investigate the impact of the different promotors on 5OH-Trp incorporation efficiency, the α-SN Tyr39Trp was cloned into pET26b plasmid behind a T7*lac* promotor and the expression was made varying the IPTG concentration in both induction stages.

The results presented in Table 2 clearly show that the change from the *T7plain* promoter to the *T7lac* increased the incorporation yield of 5OH-Trp into α-SN^{39}. The percentage of α-SN^{39}5OH-Trp

Table 1
Percentage of 5OH-Trp incorporation in the purified α-SN^{39} samples. The effect of ammonium sulfate 400 mM in the first induction step was evaluated in the 5OH-Trp incorporation yield. After wash, no salt (ammonium sulfate) was added in the induction second step. Vector pT7-7 (T7*plain* promoter).

Expression 1° step	IPTG 1mM/	IPTG 1 mM + (NH4)2SO4 400mM/
2° step	+5OH-Trp IPTG 0 mM	+5OH-Trp IPTG 0 mM
% of α-SN^{39}5OH-Trp		
	27 ± 3	**89** ± 4

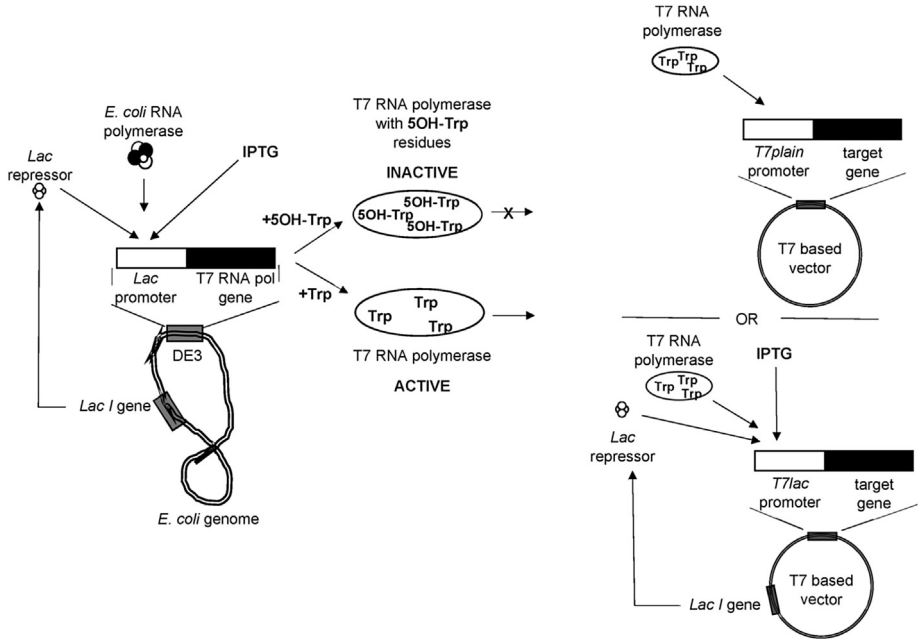

Fig. 3. Scheme representing a T7 RNA polymerase based expression system of a target gene in *E. coli* as the expression host. The results presented in Fig. 1 indicate that when 5OH-Trp (5HW) residues are incorporated in the T7 structure there is no expression of the target gene, probably because this T7 with 5OH-Trp (5HW) residues is inactive. There are 19 codons for Trp (W) residues in the T7 gene. Expression system with a vector containing the T7*plain* (right-upper panel) in comparison with the control of an expression system with a vector containing the T7*lac* (right-lower panel).

Table 2
T7*plain* x T7*lac* and the influence of IPTG concentrations on the 5OH-Trp incorporation yield in the purified α-SN^{39}. Percentage of 5OH-Trp incorporation in the purified α-SN samples obtained by the 2 step induction protocol.

Promoter	T7*plain*	T7*lac*	T7*lac*	T7*lac*	T7*lac*	T7*lac*	T7*lac*	T7*lac*
IPTG 1° step/ 2° step	1mM/ 0 mM	1mM/ 0 mM	1mM/ 0.5 mM	1mM/ 1 mM	0.5mM/ 1 mM	0.5mM/ 0.5 mM	0.5mM/ 0.25 mM	0.5mM/ 0.125 mM
% of α-SN^{39}5OH-Trp	27 ± 3	59 ± 3	73 ± 2	72 ± 1	86 ± 2	84 ± 1	89 ± 4	85 ± 5

detected in the samples increased from 27% to 59%. Increasing the IPTG concentration in the second induction step further improved the α-SN^{39}5OH-Trp/α-SN^{39}Trp ratio due to higher amounts of 5OH-Trp labeled α-SN^{39}, in contrast when compared with the T7*plain* promoter results (Table 2). Lowering the IPTG added in the first step to 0.5 mM and changing the IPTG at the second step reliable incorporation efficiencies of almost 90% were obtained (Table 2). But, reducing even more the IPTG concentration up to 0.25 mM at the first step the expression yield was so low that we were not able to get reliable peak area from the chromatograms.

Few reports describe a high incorporation rate of 5OH-Trp in proteins using T7based vectors [25–27]. Interestingly, the BL21(DE3)pLysS *E. coli* Trp auxotroph strain used produces lysozyme, which inhibits the T7 RNA polymerase [28]. As these researchers used a protocol similar the simple protocol we initially tried to use, we can conclude that, in this system, probably the basal production of T7 RNA polymerase during bacterial growth was inhibited by the presence of the lysozyme. It consequently helped to decrease the basal production of the target protein till the wash and change of the medium containing the 5OH-Trp.

Probably the best expression parameters - IPTG concentration/induction time/temperature - will vary for each target protein. Therefore, it may be necessary some small scale expression tests as the presented here to search for the best incorporation efficiency.

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