

COMBINING HETEROLOGOUS BACTERIAL EXPRESSION SYSTEM WITH AFFINITY CHROMATOGRAPHY PURIFICATION TO OBTAIN NATIVE MOUSE TYROSINASE

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Abstract

Tyrosinase is a type 1 transmembrane protein, with a pivotal role in melanin synthesis within melanocytes. Tyrosinase is also upregulated in melanoma cells and for this reason it is used as a conventional biomarker in melanoma diagnostic. Moreover, many scientific reports present tyrosinase as a well-known autoantigen capable to elicit the immune system and as a consequence, one of the main goals in melanoma therapy is to develop methods to increase tyrosinase immunogenicity. Among the experimental approaches in achieving this goal is the manipulation of the protein *in vitro*, followed by studies on animal models. Here we report expression in a bacterial system of a tagged tyrosinase from which the protein is further purified. It is known that foreign transmembrane proteins often aggregate and form inclusion bodies when they are expressed in bacteria because of their highly hydrophobic aminoacids that span the membrane bilayer. Protein extraction from these structures is usually done using denaturant agents. We were able to express mouse tyrosinase without the signal sequence and to co-purify in non-denaturant conditions this protein. Sequence confirmation of the purified product was obtained using mass-spectrometry and Western blotting.

Rezumat

Tirozinaza este o proteină membranară de tipul 1, cu un rol important în sinteza de melanină la nivelul melanocitelor. S-a constatat că tirozinaza prezintă o expresie crescută în celulele de melanom, motiv pentru care este folosită ca biomarker convențional în diagnosticul melanomului. Mai mult, date din literatură raportează tirozinaza ca un cunoscut autoantigen, capabil să stimuleze sistemul imunitar și în consecință, unul din scopurile principale în terapia melanomului este dezvoltarea metodelor care cresc imunogenicitatea tirozinazei. Printre abordările experimentale necesare atingerii acestui scop se numără manipularea *in vitro* a proteinei, urmată de studii pe modele animale. În această lucrare raportăm expresia în sistem bacterian a tirozinazei fuzionată cu glutatión S-transferaza (GST), urmată de purificarea acesteia. Este cunoscut faptul că proteinele transmembranare agregă și formează corpi de incluziune când sunt exprimate în sistem bacterian datorită aminoacizilor hidrofobi inserați în bistratul lipidic. Extracția proteinelor din aceste structuri insolubile se face de obicei cu agenți denaturanți. În acest articol raportăm expresia tirozinazei de șoarece, fără secvența semnal, într-un sistem bacterian și co-purificarea acesteia în condiții non-denaturante. Confirmarea secvenței proteinei purificate a fost realizată prin spectrometrie de masă și Western blotting.

Keywords: tyrosinase, mass-spectrometry, Western blotting

Introduction

Although tyrosinase is constitutively found in melanocytes where it catalyses the first two steps in melanin biosynthesis, it is also a biomarker in cancer melanoma, as it is an overexpressed protein in melanoma cells [8]. Sequence analysis of tyrosinase from various species showed that it is a well conserved copper containing enzyme with a high aminoacids identity (more than 80 %) between mouse tyrosinase (msTyr) and human tyrosinase (huTyr) [1, 9]. Both tyrosinases are transmembrane proteins with a large luminal domain and a short cytosolic tail. The luminal domain of tyrosinase

contains the N-terminus signal peptide (SP), two cysteine (CYS) rich domains, and two binding sites for copper atoms. The copper atoms are essential for the enzyme activity and the cytosolic tail encompasses sorting signals for the endo-lysosomal compartment [4, 17]. Human tyrosinase folding is an unproductive process and the fraction of incorrectly folded polypeptides is targeted for degradation through the ubiquitin-proteasome system or endo-lysosomal compartment that can generate antigenic peptides. The peptides resulted by proteolysis include either 9 aminoacids structures that are recognised by the major histocompatibility complex I (MHCI) [9] or 12-16

aminoacids structures, recognised by major histocompatibility complex II (MHCII) [26]. These MHC complexes mediate the transport of antigenic peptides to the cell surface for presentation to cytotoxic T cells (CTLs) and thus elicit the immune system. Recognition of antigenic peptides by cytotoxic T cells initiates an immune response that leads to destruction of tumour cells; for this reason antigenic peptides are analysed in terms of antitumor immunity and represent important therapeutic targets. This is a new biomedical approach alongside other antitumoural therapies based on treatment with inhibitors for cellular degradation compartments [21].

The immune response of xenogeneic tyrosinases has been extensively reported, with human tyrosinase being a successful canine vaccine [10,26]. To understand the immunogenicity of self-*versus* xenogeneic tyrosinase antigens, msTyr sharing high homology with huTyr is an important protein for studies in mouse model. Moreover, tyrosinase and proteins from tyrosinase family are validated biomarkers for identification and diagnose of the melanoma at early stages, therefore production of specific antibodies against these proteins is currently of highly medical interest.

Here, we report the heterologous expression and affinity purification of mouse tyrosinase (msTyr) from *E. coli* bacterial system, a convenient source for the production of recombinant proteins in biotechnology and bio-medical research fields. We were able to co-purify msTyr in non-denaturant conditions, with a yield estimated at approximately 75 ng/mL of bacterial culture. Sequence expression and purification was verified by Western blotting and mass spectrometry.

Our results suggest that the bacterial expression system can be used for large scale production of msTyr necessary for immunogenicity studies of modified proteins or to obtain conformational anti-tyrosinase antibodies.

Materials and Methods

Strains, plasmids and reagents

E. coli XL1 blue and *E. coli* expression strains (BL21 DE3, Rosetta, and RIL) were purchased from Stratagene/Novagen. Glutathione Sepharose 4B beads, PreScission protease and the expression vector pGEX6P1 were purchased from GE Healthcare. The primers were synthesized at HVD Heidelberg, Germany. The restriction enzymes, DNA polymerase and T4 DNA ligase were purchased from New England Biolabs and the DNA extraction kit was purchased from Qiagen. Isopropyl β -D-thiogalactopyranoside (IPTG), copper chloride, dihydroxyphenylalanine (DOPA), lysozyme, magnesium chloride and all other

chemicals used for mass spectrometry were purchased from Sigma Aldrich, except trypsin which was purchased from Promega. Protease inhibitors were from Roche, rabbit anti-tyrosinase Pep7 antibodies were a gift from Prof. V. Hearing/USA. The DNA ladder was from Ferment as and the protein molecular weight marker from Thermo Pierce.

Cloning of mouse tyrosinase into pGEX6P1 for expression in E. coli

The cDNA fragment encoding for mouse tyrosinase protein without the signal sequence was amplified by polymerase chain reaction (PCR) based on the complementary DNA (cDNA) of pHDmcTyr (gift from Dr. Günther Schüts), using the following primers: Forward

5'-CAATGGATCCTGTGCTCCTCTAAGAAGCTGTGG and Reverse

5'-AATTGCCGCCGCTCACAGATGGCTCTGATACAGCAAG

(the underlining indicates the *Bam*HI and *Not*I recognition sequence, respectively). Both, the PCR product and the pGEX6P1 vector were subjected to enzymatic digestion with *Bam*HI and *Not*I, then, following ligation in the presence of T4 ligase, the plasmid was transformed into XL 1 blue super competent cells, plated on agar medium containing 100 ug/mL Ampicillin (Amp) and grown at 37°C. This recombinant plasmid enables coding of a tyrosinase fusion protein with the Glutathione S-transferase (GST) tag. Positive colonies were selected by digestion with *Bam*HI and *Not*I, and one positive clone was selected for sequencing to verify the frame and insert integrity. This clone was used for further experiments.

Optimization of the GST-Tyr fusion protein expression

Colonies of *E. coli* BL21 DE3, Rosetta and RIL, harbouring the recombinant plasmid were inoculated into 5 mL Luria Bertani Broth (LB) media supplemented with 100ug/mL Amp and grown at 37°C overnight (ON) with agitation. Next day, the starter culture was diluted 1:100 in fresh LB media supplemented with Amp and incubated at 37°C up to a relative optical density of OD₆₀₀ = 0.5-0.6, when IPTG was added to induce the expression of the fusion protein. Initial selection of the optimal strain for protein expression was performed by induction with 1mM IPTG and incubation at 37°C for approximately 16 h. 1 mL aliquots of non-induced and induced culture were harvested by centrifugation at 5000 x g, for 10 min at 4°C. The pellets were resuspended in SDS-PAGE loading buffer 1x and boiled at 95°C for 5 min. The samples were resolved on 8% SDS-PAGE and the gel was stained with Coomassie Blue R-250. Further tests to enrich the fusion protein into soluble fraction were conducted on BL21 RIL strain using various conditions as: 0.1, 1 and 2 mM IPTG concentrations, harvest at 3h, 6h and 16h, grow at 25°C and 30°C,

and incubated in the presence of 1 % glucose, 0.1 mM copper chloride and 0.1 mM DOPA, respectively.

Large scale expression and purification of the recombinant mouse tyrosinase

MsTyr- pGEX6P1 was transformed in *E.coli* RIL and a starter culture was prepared in LB media supplemented with 100 µg/mL Amp. Next day, the starter culture was diluted 1:100 in 4L of culture media, grown to an OD_{600 nm} of 0.5-0.6, and induced with 0.1 mM IPTG for additional 6 h. The bacterial cells were harvested by centrifugation at 5000 x g, 4°C for 10 min. The pellet was incubated 30 min on ice with the lysis buffer (1.5 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.4 supplemented with lysozyme (1mg/mL), DNase (30ug/mL), 15 mM MgCl₂, 1.5 % Triton X-100 and protease inhibitors (1x)) and the cells were disrupted using a French press (Thermo Fisher Scientific). The homogenate was centrifuged at 20000 x g for 30 min at 4°C, and the supernatant, was incubated with the pre-equilibrated Glutathione Sepharose 4B resin (1:1500, V:V). The beads were washed three times with lysis buffer. To check the specific binding of fusion protein to the resin, an aliquot of the beads were eluted with reduced glutathione solution (50 mM Tris-HCl and 10 mM GSH, pH 8.0), for 15 min at Room Temperature (RT). The protein was purified by incubation of the remaining msTyr bound beads with PreScission protease (3C-Protease with GST tag) for 16 h at 4°C, according to manufacturer's protocol.

Mouse tyrosinase identification using Western blotting (WB) and mass spectrometry (MS)

For WB experiments the samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. This was blocked in 10 % skimmed milk in phosphate buffer saline (PBS), at RT for 1h and subsequently incubated with 1:1000 (V:V) diluted rabbit α-tyrosinase antibodies Pep7 in 5 % milk, PBS-Tween 20 0.1 % for 1h at RT. The secondary antibodies HRP (Horseradish Peroxidase) -coupled, were incubated for 1h in the same solution and the identification was assessed by incubating the membrane with luminol for 2 min at RT.

For MS analysis, the purified protein and the fraction of resin-bound proteins were separated in a polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Selected bands were sliced and the protein-containing bands were processed according to a previously described in gel digestion protocol [11]. Briefly, the gel fixed proteins were reduced by incubation with 10 mM dithiothreitol (DTT), at 56°C for 45 min, then washed and alkylated with iodoacetamide (IAA), for 45 min at RT. The dried gel pieces were incubated with 6.25 ng/µl trypsin ON at 37°C. The peptides were extracted twice using 5 % formic acid (FA) and 50 % acetonitrile (ACN) and once using 5 % FA + 95 %

ACN. The fractions from each extraction were combined, subjected to vacuum dry and kept at -20°C until further use. Before analysis, the samples were resuspended in 20 µl mobile phase A (0.1 % FA + 2 % ACN) and injected into a nano-liquid-chromatograph (nLC) (Proxeon Biosystems) coupled online with an LTQ (Linear Trap Quadrupole) Orbitrap Velos Pro mass spectrometer (Thermo Fischer Scientific). The peptides were separated first by passing the sample to a C18 trap column (2 cm x 100 µm, Proxeon Biosystems) to concentrate and desalt the peptides and then to an analytical C18 column (10 cm x 75 µm) connected online to a nanoelectrospray (nES) stainless steel emitter (Proxeon Biosystems). A 90 min gradient of 2-30 % mobile phase B (0.1 % FA + 98 % ACN) was used for the elution of the peptides from the analytical column. The MS instrument was operated by the acquisition of an initial survey scan between 300 and 1800 m/z at a resolution of 60000 (m/z 400), followed by the fragmentation of the 5th most abundant ions +2, +3 or higher charges (top 5 method) selected from the first scan, with Collision Induced Fragmentation (CID). Dynamic exclusion was enabled with a repeat count of 1, exclusion duration of 60 s and an exclusive relative to the reference mass of 10 ppm.

The acquired raw files were searched with the SEQUEST algorithm integrated into Proteome Discoverer v1.4 against a custom database containing the proteins expressed by *E. coli* strain B (BL21-DE3) to which the recombinant sequence of mouse tyrosinase and GST from *Schistosoma japonicum* were added. A second search against a decoy-database containing the reversed sequences was performed to estimate the false discovery rate (FDR). The following settings were used for the search procedure: precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.5 Da, trypsin as the enzyme used for the digestion with maximum two missed cleavage sites (full tryptic), carbamidomethylation on cysteine residues as a static modification and methionine oxidation as a dynamic modification. The results were further filtered by considering only peptides with a precursor mass tolerance of maximum 5 ppm and 99 % confidence (Peptide spectrum match (PSM) level)).

Results and Discussion

Construction of the recombinant plasmid

Similar to the human homolog, msTyr contains at the N-terminus a putative signal peptide of 23 amino acids residues, which are mostly hydrophobic and are removed from the mature tyrosinase upon entering endoplasmic reticulum (ER) [17]. To avoid intracellular protein aggregation, for heterologous expression of tyrosinase we cloned

the cDNA encoding for msTyr without the signal peptide into an *E. coli* expression vector (Figure 1A). The cDNA was amplified by PCR using a pair of specific primers and wild type tyrosinase as a template and cloned into *Bam*HI/*Not*I site of the pGEX6P1 vector. Screening for the positive colonies by digestion of the isolated plasmids with *Bam*HI and *Not*I showed the presence of the expected insert (1539 base pairs (bp)) in 5 out of the 7 plasmids (Figure 1B).

Table I

Predicted biochemical properties of the mouse tyrosinase sequence and of the GST-fusion protein

	# Aa.	Molecular mass (kDa)	pI
msTyr	510	57.85	5.85
fusion protein (GST-msTyr)	741	84.64	5.89

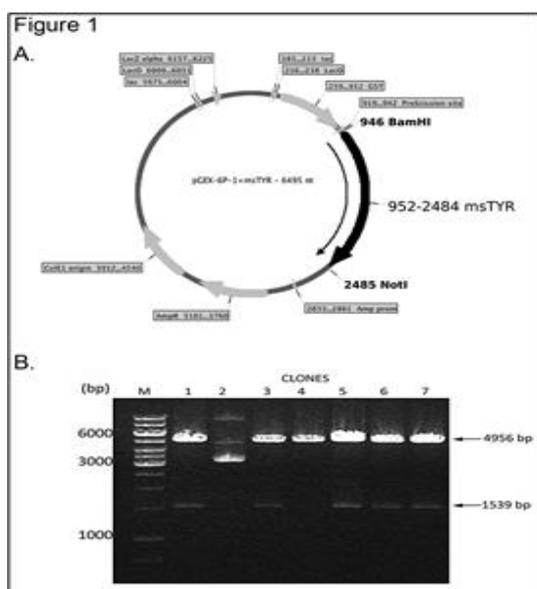


Figure 1. Sequence features of the recombinant plasmid msTyr-pGEX6P1 and positive colony selection of mouse tyrosinase (accession no. P11344).

A. Schematic representation of the recombinant plasmid msTyr-pGEX6P1 with the insert of 1539 bp and the cloning sites highlighted, drawn using Serial Cloner 2.6.

B. Digestion of the plasmids with *Bam*HI and *Not*I confirmed insert presence in five positive colonies (col. 1, 3, 5, 6 and 7).

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The plasmid isolated from one positive colony (clone 3) was sequenced to confirm the integrity of the tyrosinase gene and further used for protein expression. From this vector, msTyr could be expressed in *E. coli* as a fusion GST-Tyr protein upon activation of the *tac* promoter with IPTG [15]. The physicochemical properties, of msTyr and the fusion protein were predicted using Serial Cloner 2.6 and they are detailed in Table I.

Expression of the GST-Tyr fusion protein

In order to establish the optimum conditions for expression of the fusion protein BL21 DE3, Rosetta and RIL *E. coli* strains were tested, under identical expression conditions. The expression of recombinant protein was successfully induced in these strains and showed an apparent molecular mass of about 84 kDa, which is in agreement with the predicted size deduced from the amino acids sequence. Production of the recombinant protein was low in BL21 DE3 and Rosetta strains as ascertained by Coomassie staining of the proteins from total lysates (Figure 2A). A major protein band was observed in the total lysates of RIL cells which reflects efficient translation of GST-Tyr due to the extra copies of the *argU*, *ileY*, and *leuW* transfer RNA (tRNA) genes (Figure 2A lane 6) [2].

Under initial growing conditions most of the recombinant protein was found in insoluble fraction and only a small amount in the soluble cell fraction. Probably the large quantity of proteins that accumulate in inclusion bodies are insoluble aggregates formed by misfolded or incomplete folded proteins, due to higher amounts of protein being processed in a short time. Since protein recovery from inclusion bodies requires chaotropic agents that could hamper down-stream chromatographic steps of protein purification [7] we focused on enhancing the yield of GST-Tyr in the soluble fraction by optimizing various growth conditions [20]. The bacterial cell pellets corresponding to each treatment were lysed, centrifuged and fractions of supernatant and pellet were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Analysis of the gel revealed that the overnight incubation at 25°C with 1 mM IPTG expressed the highest amount of soluble GST-Tyr (Figure 2B lane 7). Addition of glucose, which promotes protein folding by partially repressing the *lac* promoter [13], did not significantly increase production of soluble recombinant protein (Figure 2B, compare lane 7 and 9). It was previously shown for human tyrosinase that the presence of its substrate, changes its state from misfolded to native form, and in addition, the presence of cofactors is often required for the enzyme to achieve its native conformation [5]. Therefore we added tyrosinase substrate (DOPA) and cofactor (copper chloride) to the culture media and indeed, as observed in Figure 2C, expression of the recombinant

protein in the soluble fraction was enhanced following their addition.

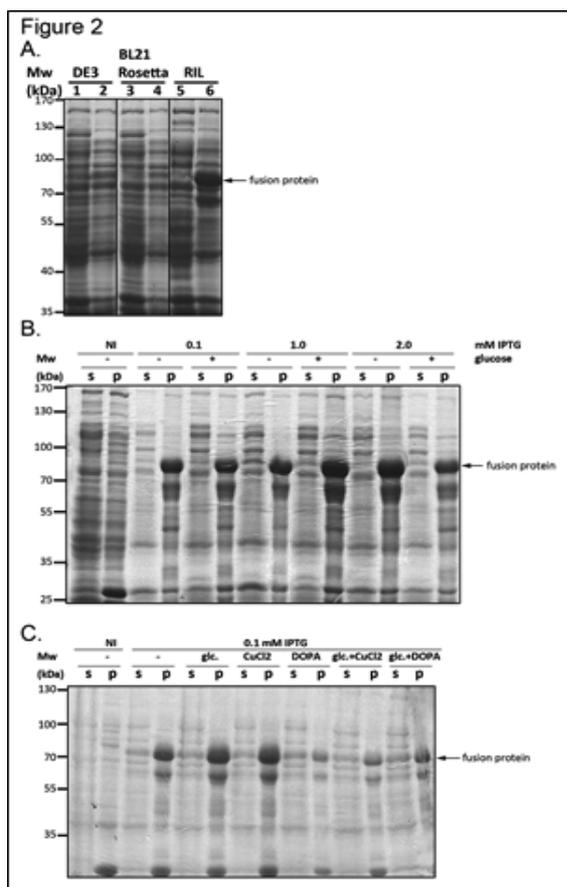


Figure 2.

SDS-PAGE analysis of *E. coli* strains expressing GST-Tyr fusion protein under various growing conditions. A. Expression of GST-Tyr fusion protein in BL21 DE3, Rosetta and RIL strains. The bacteria strains were grown at 37°C and protein expression was induced with 1mM IPTG for approximately 16 h. Equal volumes of total cell lysates from induced (lane 2, 4, and 6) and non-induced cells (lane 1, 3, and 5) were separated in a polyacrylamide gel, followed by protein staining with Coomassie Brilliant Blue R-250.

B. Optimization of fusion protein solubility in BL21 RIL strain. 0.1, 1 and 2 mM IPTG, respectively, were added to the bacterial culture supplemented or not with 1 % glucose. The bacteria pellets were lysed, centrifuged and equal volumes of the supernatant (s) and pellet (p) fractions were resolved in a polyacrylamide gel for detection of the proteins using Coomassie Brilliant Blue R-250.

C. Protein expression in the soluble fraction was tested in the presence of tyrosinase folding agents..

Bacterial cells were grown at 25°C for 6 h and protein expression was induced with 0.1 mM IPTG in the presence of indicated additives. Cells were lysed; centrifuged and equal volumes from supernatant (s) and pellet (p) were resolved by SDS-PAGE. Proteins were detected using Coomassie Blue R-250 staining.

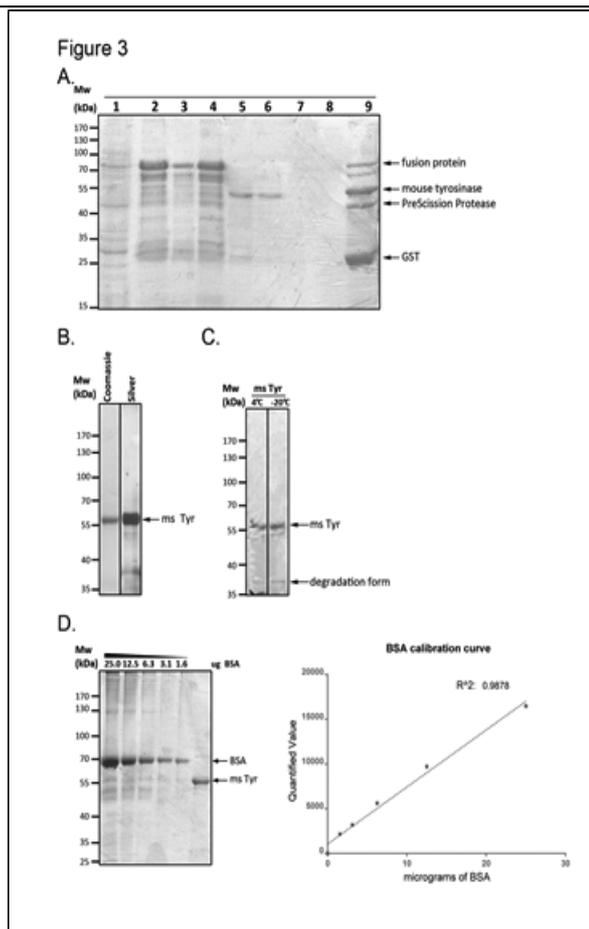


Figure 3.

Purification of the recombinant protein, stability test of the purified product and estimation of the yield

A. The fusion protein was purified by affinity chromatography using GSH Sepharose beads and the fractions corresponding to unbound proteins (lane 1), proteins bound to the beads (lane 2), proteins eluted with elution buffer (lane 3), proteins retained on the beads after elution (lane 4), the supernatant following incubation with PreScission Protease (lane 5 and 6) and the proteins retained on the beads after PreScission digestion (lane 9) were separated using SDS-PAGE and stained with Coomassie Blue R250.

B. Equal amounts of the purified protein were loaded in a polyacrylamide gel and the separated proteins were detected using either Coomassie Brilliant Blue R-250 (left panel) or silver nitrate (right panel) to assess the purity of the purified product.

C. Equal amounts of the purified protein were kept several days at 4°C or subject to multiple freeze-thawing cycles at -20°C. The tyrosinase integrity was monitored by SDS-PAGE and Coomassie staining.

D. A calibration curve obtained by serial dilutions of BSA (1.6-25 µg) was loaded in a polyacrylamide gel, stained with Coomassie and used to estimate the concentration of the purified protein (left panel). Scatter plot of the BSA calibration curve and estimation of the protein content (right panel).

Protein purification, stability and yield of purification

The recombinant protein was purified using Glutathion Sepharose affinity chromatography from the soluble fraction as described in Material and Methods section. This allowed us to obtain a concentrated fraction of the fusion protein that was subsequently cleaved with PreScission protease. SDS-PAGE analysis of the protein content showed that most of the fusion protein bound to the resin (Figure 3A lane 2) was efficiently cleaved under our optimized conditions (Figure 3A, lane 5, 6). However, analysis of the purified fraction separated in low concentration polyacrylamide gels that give a higher resolution, revealed the presence of two protein bands with a molecular mass similar with that expected for msTyr, ~ 58 kDa (Figure 3B, lane1). Silver nitrate staining, a protein identification method with a higher sensitivity [19] than Coomassie staining, did not revealed any apparent protein contaminants (Figure 3B, lane2).

Using this protocol we estimated a yield of ~ 250-300 µg of tyrosinase protein from 4 L of bacterial cell culture (using Bicinchoninic Acid based Assay - BCA and the calibration curve from Figure 3D). We also observed that the protein is more stable after the incubation at 4°C for a few days, compared with a freeze/thawing cycle at -20°C (Figure 3C).

Identification of the purified protein by Western blotting and mass spectrometry

Western blot analysis confirmed the presence of the fusion protein with possible degradation forms (Figure 4A, lane 2 and lane 4). Several bands were also observed at a higher molecular mass (Figure 4A, lane 2) when samples were separated in native conditions (no boiling and no DTT). These bands could not be observed in reducing conditions indicating that probably they are multimeric proteins linked by disulphide bonds or protein aggregates. To confirm the identity of msTyr serial dilutions of the purified fraction were loaded alongside HEK293T Human Embryonic Kidney) lysate as a negative control. Although this control lysate did revealed the presence of a non-specific band at a similar molecular mass with that expected, the variation of the signal for the second band was according to the dilutions used in the purified fractions. This inconclusive result from Western blot analysis of the purified fractions prompt us to use mass spectrometry, a sensitive method for protein sequence confirmation and small molecules analysis [24, 25].

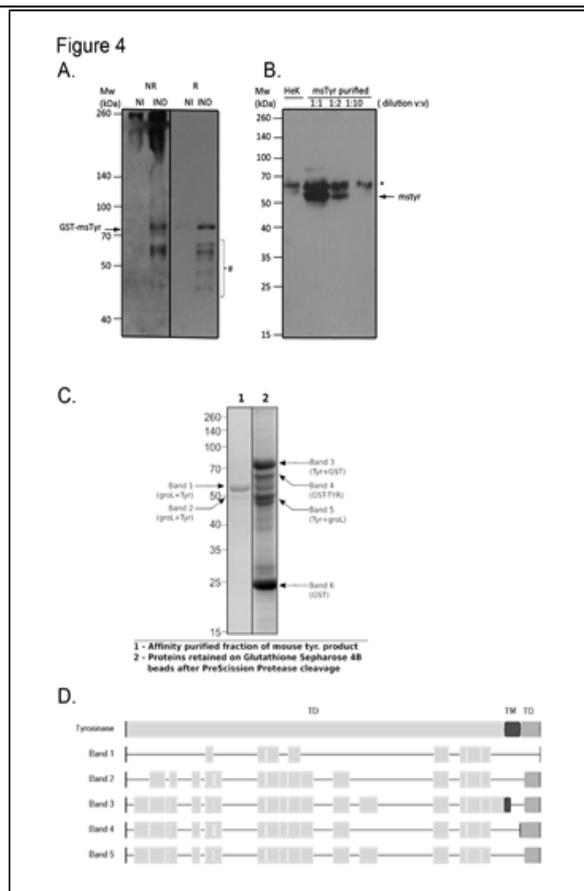


Figure 4.

Protein identity confirmation using Western blot and mass spectrometry

- A. Non induced (NI) and induced (IND) bacterial lysates were loaded in 8% polyacrylamide gel in nonreduced (NR) and reduced (R) conditions, separated and transferred on nitrocellulose membrane and probed with anti tyrosinase pep 7 antibodies. # denotes possible degradation forms of GST-Tyr under the expression or purification process.
- B. Three dilutions of the purified tyrosinase and a negative control (HEK cell lysate) were separated by SDS PAGE, transferred on nitrocellulose membrane and probed with anti-ms tyrosinase antibody (pep7). The asterisk (*) denotes a protein that cross-reacted with the pep7 antibody.
- C. The supernatant following a partial cleavage with PreScission protease and the proteins retained on the sepharose beads were resolved by SDS-PAGE and the resulting gel was stained with Coomassie Brilliant Blue R250. Designated bands were cut out from the gel and subjected to in gel digestion and MS analysis.
- D. Sequence coverage of tyrosinase in the five bands analyzed using LC-MS/MS and protein topology according to Uniprot database. TD denotes topological domains of the protein and TM the transmembrane region (graphical representation using Caititu [3]).

We conducted a partial cleavage experiment followed by SDS-PAGE of the affinity purified fraction and the Sepharose retained proteins. The bands of interest were cut out from the gel and the proteins were digested with trypsin. Liquid Chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis of the purified fractions (Figure 4C, lane 1, band 1 and band 2), confirmed the presence of tyrosinase in both bands (Table II). However the results also revealed the presence of a bacterial ~60 kDa chaperone in each band. This is not surprising considering that folding of transmembrane proteins, as tyrosinase, is known to be assisted by cellular chaperones [14, 22].

Since both affinity purified species (lane 1, band 1 and band 2) contained tyrosinase, one possibility is that the faster migrating protein is a degradation form of tyrosinase. Sequence coverage comparison of tyrosinase (Figure 4C, compare band 1 and band 2) did reveal some peptides that were found only in the faster migrating band of the purified protein. However, these additional peptides found in the second sample were randomly distributed across the sequence suggesting that it could be identified only on the purely basis of different protein content between the two bands. Although alternative spliced transcripts of tyrosinase were described in eukaryotic systems [12, 16, 18], this analysis also excluded also this possibility.

Table II

Top two scoring proteins identified by SEQUEST in each band subject to in gel digestion and LC-MS/MS analysis

	Gene name	% coverage	Score
Band 1	groEL	85.22	6463.09
	Tyr	40.78	255.52
Band 2	groEL	62.04	950.12
	Tyr	51.18	897.72
Band 3	Tyr	70.00	772.62
	GST	66.51	413.82
Band 4	Tyr	61.57	640.74
	GST	47.71	272.04
Band 5	Tyr	64.90	987.12
	groEL	44.89	326.05
Band 6	GST	57.34	1249.48
	rpsC	35.62	185.21

Since we also observed two expression bands for the fusion protein, probably due to bacterial endoproteases activity along the expression process [23], we hypothesized that the double band pattern of tyrosinase is a result of the initial fusion protein cleavage. To evaluate this idea we compared the sequence coverage of fusion tyrosinase found in the fraction of proteins retained on Sepharose beads following partial cleavage with PreScission Protease (Figure 4D, lane 2 bands 3 and 4). LC-

MS/MS analysis confirmed the presence of tyrosinase in these bands, with no significant differences between the sequence coverage, except for a few peptides from the transmembrane region (Figure 4D), reinforcing our hypothesis. This may imply that the faster migrating band from lane 1 (band 2) may be provided after GST cleavage of the second band of GST-Tyr (band 4). Even more, tyrosinase was also identified in band 5. This could be explained by the observation that the protein precipitates following removal of the GST tag, since one of the main role of GST is to facilitate protein folding [6]. As expected, GST, the fusion partner in the expression process was identified in band 6 with a high sequence coverage (>50%, in Table II), although it was also found in bands 3 and 4, but with a lower sequence coverage (data not shown).

Conclusions

We were able to express mouse tyrosinase in a heterologous bacterial system with optimized growth conditions for an increased expression in the soluble fraction.

We have obtained the protein in non-denaturing conditions making it compatible with further immunogenicity testing.

The identity of the protein was confirmed using western blotting and nanoflow high performance liquid chromatography coupled with nano-electrospray ionization mass spectrometry.

Taken together, these data show that tyrosinase, a transmembrane protein, can be isolated from the bacterial soluble cell fraction in complex with a folding chaperone (GroEL), which is compatible with further structural modifications studies.

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