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Production of soluble, active acetyl serotonin methyl transferase in *Leishmania tarentolae*

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ABSTRACT

N-acetyl serotonin methyl transferase (ASMT) is the last enzyme in the melatonin synthesis pathway. Evidence linking autism-related disorders with disorders of melatonin metabolism, and, more specifically, with mutations of the gene encoding ASMT, prompted us to investigate the properties and localization of this enzyme. As a first step, we undertook to overproduce the protein in a recombinant host. Early attempts to produce ASMT in recombinant *Escherichia coli* yielded only insoluble and heavily degraded material. However, recombinant ASMT (rASMT) could be produced in soluble, active form and purified in milligram amounts when the gene was cloned and expressed in *Leishmania tarentolae*.

Introduction

N-acetyl serotonin methyl transferase (ASMT)[3] is the enzyme responsible for the last step of the synthesis of melatonin, a key regulator of circadian and seasonal rhythms [1]. Abnormal melatonin concentrations have a dramatic effect on human behavior and have been connected to autism-related disorders [2–4]. Recently, Melke et al. found a significant correlation between autism spectrum disorders and mutations affecting the gene encoding ASMT [4]. In order to provide tools for further investigating the structure and function of ASMT, as well as its localization, we sought to produce and purify the enzyme from a suitable recombinant host. Attempts to synthesize ASMT in recombinant *Escherichia coli* yielded only insoluble and degraded material, in spite of various attempts to improve expression conditions such as lowering cultivation temperature or co-expressing chaperones (data not shown). This prompted us to try the *Leishmania tarentolae* host-vector system originally developed by Breitling et al. [5]. Indeed, cloning and expression of human ASMT cDNA in *L. tarentolae*, a lizard parasite harmless to humans, enabled the production and purification in milligram amounts of soluble, active enzyme.

Materials and methods

Cultivation of L. tarentolae

The *L. tarentolae* LEXSY P10 host strain was grown at 26 °C in BHI medium (Jena Bioscience) containing 5 μg/ml hemin, 50 units/ml penicillin and 50 μg/ml streptomycin. When needed, hygromycin (50 μg/ml) was added to select recombinant clones. Static cultures were maintained at 26 °C in TC25 flasks containing 10 ml medium, and diluting suspensions 10–20-fold into fresh medium twice weekly. The number of cells was determined using a Malassez hemocytometer. For the production of rASMT, 1 l shaken cultures of the transfected strain were grown in Fernbach flasks. The flasks, containing 900 ml prewarmed BHI medium, were inoculated with 100 ml mid-late growth phase precultures (OD_{600} = 4–6) and shaken at 140 rpm, 26 °C in the dark for 44 h before harvesting. The final OD_{600} was 8–8.5.

Cloning of the cDNA encoding ASMT

RNA was extracted from lymphoblastoid cell lines with the TOTALLY RNA kit (Ambion), following the manufacturer's instructions. mRNA was reverse transcribed with SuperScript II Reverse Trancriptase (Life Technologies). The *ASMT* coding sequence was amplified by PCR with Taq Hotstar (Qiagen) and the *ASMT*-specific primers, 5′ATG: CACCATGGGATCCTCAGAGGACCA and 3′STOP:

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[3] *Abbreviations used*: ASMT, acetyl serotonin methyl transferase; rASMT, recombinantacetyl serotonin methyl transferase; PMSF, phenylmethyl sulfonyl fluoride; NAS, Nacetylserotonin;CHO cells, Chinese hamster ovary cells.

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GTTATTTCCTGGCTAAAATGGC. The whole PCR product was then cloned into pENTR-D-TOPO vector with a Topo Cloning Kit (Life Technologies). DNA minipreparations were performed with the PureLink Quick Plasmid Miniprep Kit (Life Technologies). A clone derived from the *ASMT* mRNA isoform without exon 6 was sequenced and controlled for the absence of mutations by comparing with the reported sequence (GenBank accession no. U11098, encoding protein no. AAA75291.1).

Cloning of the ASMT gene in L. tarentolae

The sequence of the *ASMT* cDNA was amplified by PCR, using primers ASMT-C-0001: AAAAAGATCTGCCATGGGATCCTCAGAG GACCAGGC, and ASMT-C-1035r: TTTGGTACCTTTCCTGGCTAAA ATGGCATCATAA, carrying extensions for in-frame cloning of the fragment between the *Bgl*II and *Kpn*I sites, respectively, of pLEXSY-hyg2 vector (Jena Bioscience). The resulting construct, encoding the full sequence of ASMT fused to a C-terminal His_6 tag, was transformed into *E. coli* TOP10 (Invitrogen). After verifying the sequence of the insert, a linear *Swa*I fragment containing the *ASMT* insert was excised from the plasmid and purified by agarose gel electrophoresis. For electroporation, about $1.5–2 \times 10^8$ *L. tarentolae* cells from a 2 ml, 2 day old preculture were centrifuged and resuspended in 1 ml ice-cold Cytomix electroporation buffer containing 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$, adjusted to pH 7.6 with KOH. Three hundred fifty micro liter of the suspension were mixed with 3 μg *Swa*I fragment and subjected to electroporation in 4 mm wide cuvettes. Two 1500 V pulses with a capacity setting of 25 μF were applied (time constant 0.3 ms), after which the cells were kept on ice for 10 min, followed by transfer into 10 ml BHI medium and incubated overnight at 26 °C. Two milliliters aliquots of the suspension were centrifuged for 5 min at 2000*g*, resuspended into 50–100 μl of BHI medium and the cells were gently plated on fresh BHI-agar plates containing 100 μg/ml hygromycin. The presence of the *ASMT* cDNA was confirmed by extracting genomic DNA from the transfectants using the DNA extraction kit provided by Jena Bioscience and performing PCR reactions with one primer hybridizing within the expression cassette and the other hybridizing to the *ssu* sequence of *L. tarentolae*.

Expression of the ASMT gene

To select the best rASMT producers, ten positive transfectants of *L. tarentolae* were grown during 48 h in 24-well plates, followed by transfer to 10 ml TC 25-flasks and cultivation for two further days, reaching an OD_{600} of 1.5–3.5. Aliquots standardized for equal cell content were centrifuged for 5 min at 3000 and resuspended in 60 μl SDS–PAGE loading buffer. The samples (20 μl) were subjected to Western blot analysis. After SDS–PAGE electrophoresis and transfer to PVDF membranes, the membranes were blocked for 30 min at room temperature in Dulbecco's phosphate buffered saline (PBS) (GIBCO®) containing 4% nonfat dry milk, followed by incubation for 2 h at room temperature in the same buffer containing anti-His tag antibody (Sigma) diluted 5000-fold. After rinsing 3–4 times in PBS, the membranes were incubated again for 1 h at room temperature in PBS containing 4% nonfat dry milk and a 10,000-fold dilution of peroxidase-conjugated goat anti-mouse antibody (AbCys, Paris), followed by rinsing 4 times in PBS. The His_6-tagged ASMT polypeptide was revealed by exposure to autoradiographic film after soaking the membranes in chemoluminescent substrate (ECL kit, Pierce) and the clones yielding the strongest 40 kDa band were chosen for further production and purification.

Purification of recombinant ASMT

Purification was performed on cell pellets from two 1 l shake flask cultures. All steps were performed on ice or at 6 °C. Cells were resuspended in 100 ml buffer A (50 mM Tris–HCl, pH 8.2, 200 mM NaCl, 5 mM imidazole, 1 mM PMSF) containing two tablets of Roche Complete™ protease inhibitor cocktail and ruptured at 110 MPa in an Aminco French pressure cell. Insoluble material was removed by centrifuging for 50 min at 40,000*g* and the supernatant was loaded on a 1.25 ml HisSelect Nickel column (Sigma) equilibrated in buffer A. After rinsing the column with buffer A, adsorbed proteins were eluted with a 20 ml gradient ranging from 5 to 300 mM imidazole in the same buffer, with a flow rate of 1 ml min^{-1}, followed by a 15 ml step at 300 mM imidazole. Fractions were analyzed by SDS–PAGE and those displaying the highest enrichment in rASMT were pooled and concentrated by ultrafiltration using Amicon Ultra-15 units with a cut-off of 10 kDa. The concentrated preparation (2 ml) was further purified on a HiLoad 16/60 Superdex 75 column equilibrated in buffer A without imidazole and eluted with a flow rate of 0.7 ml min^{-1}. Pooled fractions were concentrated by ultrafiltration, frozen in liquid nitrogen and stored at −80 °C.

The apparent size and state of aggregation of rASMT was analyzed by DLS using a WYATT DynaPro MS800 instrument.

Assay of rASMT concentration and activity

The concentration of rASMT protein was estimated from the A_{280} of the samples, with a calculated extinction coefficient of 1.1 AU g^{-1}L.

rASMT activity was assayed according to Ref. [6]. 50 μl samples containing 5 μg rASMT in 0.05 M sodium phosphate buffer, pH 7.9, were incubated (37 °C; 30 min) with 25 μl of *N*-acetylserotonin (NAS) (final concentration, 50 μM) and 25 μl [^{3}H-methyl]-*S*-adenosyl-L-methionine (final concentration 300 μM; specific activity 50 Ci/mol) in a total volume of 100 μl. Enzymatic reactions were stopped by adding 200 μl 0.45 M sodium borate buffer, pH 10, and 1 mL chloroform. The radiolabelled [^{3}H]melatonin-containing organic phase was washed twice with borate buffer. Finally, 0.8 mL samples of the organic phase were taken to dryness and radioactivity was determined using a scintillation counter. Background was calculated as the radioactive products formed in the absence of NAS. One unit of activity corresponds to the methylation of 1 nmol [^{3}H]NAS per hour.

Results

Cloning and expression of the human ASMT cDNA in L. tarentolae

The coding sequence of human *ASMT* was amplified by PCR from the corresponding cDNA isoform not containing exon 6 [7] and cloned into the pLEXSY-hyg2 vector (Jena Biosciences). The resulting construct encoded the full sequence of *ASMT* fused to a C-terminal His_6 tag. Upon transfecting *L. tarentolae*, the construct was inserted by homologous recombination into the chromosomal 18S RNA locus (*ssu*), which provided high-level, constitutive transcription of the target gene by RNA polymerase I. Ten transfectants having integrated the construct were analyzed by Western blotting to screen the best producers of His_6-tagged rASMT protein. As shown in Fig. 1, production was quite variable between different clones. Clones 2 and 3 were chosen for further studies. When followed by Western blotting, the time course of rASMT production paralleled the growth of the culture, as expected from a construct designed for constitutive expression, and most of the immunoreactive material was found in the soluble fraction (data not shown).

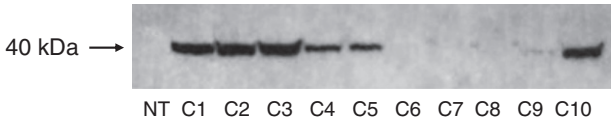

Fig. 1. Screening of recombinant *L. tarentolae* clones for the production of rASMT Cell pellets normalized for the same number of cells were collected from cultures of various hygromycin-resistant transfectants and analyzed by Western blotting as described in Methods. NT: not transfected cells; C1–C10: clones 1–10.

Addition of glucose (3 g/L) to the BHI medium enhanced neither the growth of *L. tarentolae* nor the production of rASMT (data not shown).

Purification and activity of rASMT from L. tarentolae

Immobilized Ni^{2+} affinity chromatography of recombinant *L. tarentolae* crude extracts, yielded a preparation featuring a major protein migrating as a 40 kDa species in SDS–PAGE under reducing conditions, close to the expected size of the encoded polypeptide (39.4 kDa) (Fig. 2). The eluted fraction was further purified by size exclusion chromatography. As shown in Fig. 3, highly purified protein eluted in two peaks. Dynamic light scattering analysis and calibration of the column indicated that the first peak corresponded to high molecular weight aggregates, whereas the second peak was consistent with a dimer. The latter was selected for activity analysis. The final yield of purified protein was 2.1 mg starting from 400 mg total protein present in the crude extract. The specific activity of rASMT was 1000 units/mg for the purified dimer, which corresponds to a turnover number of $1.09 \times 10^{-2}\ s^{-1}$ (Table 1).

Discussion

ASMT has been purified from pineal gland tissue of cow, chicken and rat, but, for obvious reasons, recombinant DNA technology is the method of choice to isolate and characterize the human enzyme. Ishida et al. reported the synthesis of bovine rASMT in *E. coli* [8] and CHO cells [9]. However, neither system appears as well adapted as *L. tarentolae* for purifying milligram amounts of rASMT. In the case of *E. coli*, ASMT was fused to β-galactosidase, yielding a 150 kDa polypeptide. Our own attempts to produce ASMT fused to a shorter His_6 tag in *E. coli* yielded only insoluble and highly degraded material. Production in transfected CHO cells yielded the genuine product, yet, for the best clone, the specific activity of the crude extract indicated that the amount of enzyme was tenfold less than in pituitary extracts [9]. In addition, mammalian cells are not nearly as simple to grow as *L. tarentolae* cells.

The electroporation protocol described here (see Materials and methods) was modified from the high voltage protocol suggested by Jena Biosciences in that Cytomix was substituted for BHI medium, which leads to arcing. The high voltage protocol yielded about ten times more transfectants (50 colonies/μg) than the low voltage protocol with BHI medium. We did not investigate why different transfectants displayed variable expression levels of rASMT; the variability may be due to the number of copies inserted into the *ssu* locus, or to the fact that some sites of insertion among the multiple copies of 18 S rDNA are more actively transcribed than others. At any rate, the clone chosen for high expression did not differ significantly from the wild type in terms of growth rate and biomass yield (data not shown).

Human *ASMT* mRNA occurs in three alternative splicing isoforms [7]. The longest form (form 1) contains exons 6 and 7; the intermediate form (form 2) contains exon 7 but not exon 6 and the shortest form (form 3) contains neither exon 6 nor exon 7. Exon 6 corresponds to line-1, a repetitive element not found in *ASMT* genes from other species, and it was surmised that its presence would result in the insertion of an aberrant peptide sequence in the midst of the ASMT protein, which would be detrimental to the activity of the enzyme [7]. Our own data, based upon transfection of COS cells with cDNAs corresponding to the three forms, confirm that only form 2 encodes active ASMT (unpublished results).

The sequence of ASMT comprises nine cysteine residues, which may suggest that disulfide bridge formation could be required for proper folding. However, the fact that active enzyme could be recovered from the cytoplasmic fraction of *L. tarentolae* and *E. coli* [8] argues against this possibility. Rather, the reduced state of at least some of the SH groups appears essential for ASMT activity: rat ASMT could be inactivated by disulfide exchange using disulfide-containing compounds such as cystamine and inactivation could be reversed or prevented in the presence of a reducing agent such as dithiothreitol [10].

Upon gel filtration under non-denaturing conditions, human rASMT behaved as a dimer, similar to bovine and chicken ASMT purified form pituitary tissue [11]. The specific activity of purified human rASMT reported in this study was rather low, yet it was in the same range as the values found for ASMT purified from pineal gland tissue. Thus, values ranging from 343 units/mg [6] up to 2830 units/mg [11] have been reported for bovine ASMT. In addi-

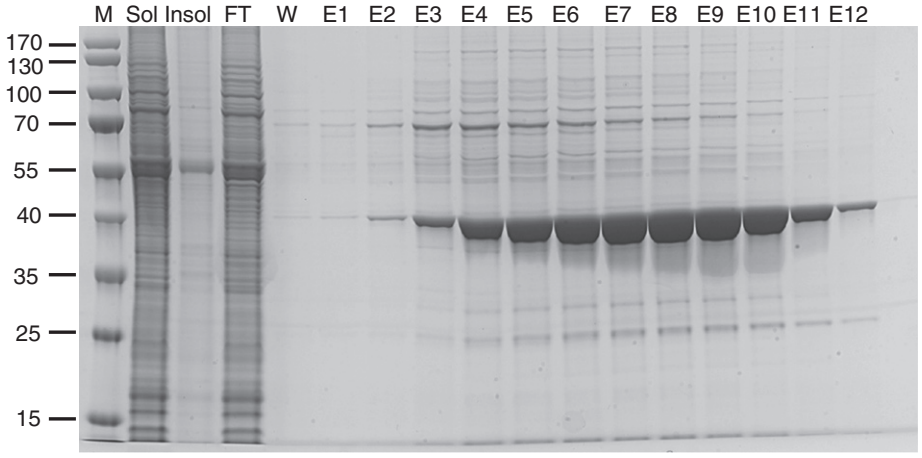

Fig. 2. SDS–PAGE analysis of fractions obtained after immobilized Ni^{2+} affinity chromatography of rASMT produced in *L. tarentolae* M, mass markers; Sol, soluble crude extract; Ins, insoluble fraction; FT, flow-through of the Ni^{2+} affinity column; W, wash fraction; E1–E12, fractions eluted with imidazole. The size of mass markers in kDa is indicated on the left. Fractions E3–E11 were pooled and concentrated by ultrafiltration for further purification by size exclusion chromatography.

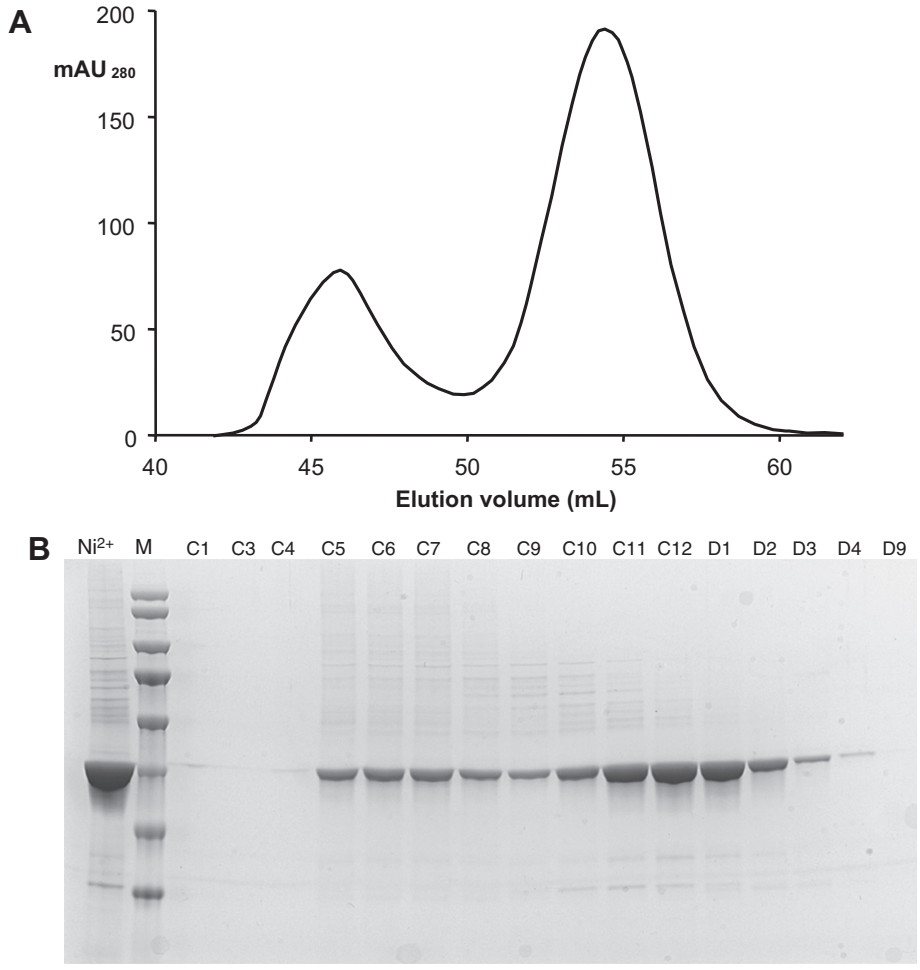

Fig. 3. Size exclusion chromatography of the rASMT fraction purified by Ni^{2+} affinity chromatography (A) Elution profile from the HiLoad 16/60 Superdex 75 column; (B) SDS–PAGE analysis of the corresponding fractions. mAU, milliabsorbance units; Ni^{2+}, pooled fraction frome the Ni^{2+} affinity column; M, mass markers (kDa); C1–D9, eluted fractions. The Figure is drawn such that lanes C5–D4 line up with the elution profile of panel A. The size of the mass markers is the same as in Fig. 2. Fractions C10–D3 were pooled and concentrated for further analysis.

Table 1
Purification of rASMT from recombinant *L. tarentolae*.

Purification step	Total protein (mg)	Purity	Specific activity (u/mg)
Crude extract	400[a]	ND[b]	ND[b]
Ni^{2+} affinity chromatography	4.1	ND[b]	ND[b]
Gel filtration	2.1	>90%	1000

Figures are given for 1 l original culture volume.
[a] As determined with the Bradford reagent (Bio-Rad) [12], with bovine serum. Albumin as a standard.
[b] ND, not determined.

tion, the activity of ASMT seems also quite variable depending on the species. Thus, in the same study, Nakane et al. [11] reported a specific activity of 2830 units for purified bovine ASMT and 11,000 units for the chicken enzyme.

Conclusion

Easy cultivation and genetic engineering of *L. tarentolae* make it an attractive alternative whenever the expression of a eukaryotic gene turns out to be problematic in bacterial hosts such as *E. coli*, due to misfolding and degradation. In the case of rASMT, the fact that the protein produced in *L. tarentolae* was soluble and active shows that it was correctly folded and intact. In addition, the protein thus obtained was crystallized and its 3-D structure was determined (A. Haouz, personal communication), further demonstrating the quality of the preparation.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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