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# Expression, purification and crystallization of human kynurenine aminotransferase 2 exploiting a highly optimized codon set

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ABSTRACT

Kynurenine aminotransferase (KAT) is a pyridoxal-5'-phosphate (PLP) dependent enzyme that catalyses kynurenine (KYN) to kynurenic acid (KYNA), a neuroactive product in the tryptophan metabolic pathway. Evidence suggests that abnormal levels of KYNA are involved in many neurodegenerative diseases such as Parkinson's disease, Huntington's disease, Alzheimer's disease and schizophrenia. Reducing KYNA production through inhibiting kynurenine aminotransferase 2 (KAT2) would be a promising approach to understanding and treating the related neurological and mental disorders. In this study we used an optimized codon sequence to overexpress histidine-tagged human KAT2 (hKAT2) using an *Escherichia coli* expression system. After a single step of Ni-NTA based purification the purified protein (>95%) was confirmed to be active by an HPLC based activity assay and was crystallized using the hanging-drop vapour diffusion method. The crystal system represents a novel space group, and a complete X-ray diffraction data set was collected to 1.83 Å resolution, and higher resolution data than for any reported native human KAT2 structure. The optimised method of protein production provides a fast and reliable technique to generate large quantities of active human KAT2 suitable for future small-molecule lead compound screening and structural design work.



## 1. Introduction

Kynurenic acid (KYNA) is produced in the tryptophan metabolic pathway and antagonises endogenous N-methyl-D-aspartate (NMDA) receptors [1]. It also acts as a non-competitive antagonist of the α7 nicotinic acetylcholine receptor, as well as an agonist of the G protein coupled receptor GPR35 [2]. KYNA is generally considered neuroprotective at normal levels [3], but fluctuations in concentration may lead to a series of mental and neurological events due to the compound's multiple activities affecting glutamatergic, cholinergic and dopaminergic neural transmission [4,5]. Indeed, abnormal levels of KYNA have been observed in many neurodegenerative diseases such as Parkinson's disease, Huntington's disease, Alzheimer's disease and schizophrenia [6]. Based on preclinical studies KYNA is involved in the pathophysiology of schizophrenia and several other psychotic and neurological disorders [7–9]. Elevated levels of KYNA have been detected in the cerebral spinal fluid (CSF) of schizophrenic patients [10,11], suggesting that the regulation of brain kynurenic acid levels, specifically a reduction in concentration in affected individuals, could provide an avenue to develop novel therapeutic strategies for relieving schizophrenia.

Kynurenine aminotransferase (KAT), that catalyses the conversion of KYN to KYNA, is a pyridoxal-5′-phosphate (PLP) dependent enzyme [12]. At present, four KAT isoforms have been identified and characterised (KAT1, KAT2, KAT3 and KAT4), and they all have been shown to possess enzymatic activity [13]. Most KYNA in the mammalian brain - that is otherwise unable to penetrate blood-brain-barrier [14] is produced endogenously by both KAT1 and predominantly by KAT2 [15]. In KAT2 knockout mice studies, the upregulation of KAT1 and KAT3 occurs in order to compensate for low levels of KYNA [16], although the inhibition of KAT2 is generally

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considered the most likely as an effective way to reduce KYNA levels in the brain. Several human KAT2 (hKAT2) inhibitors have been developed [17], most notably PF-04859989, an irreversible inhibitor of hKAT2 [18] and an orally active reversible inhibitor, BFF-816 [19]. Several *in vivo* studies using these inhibitors in rats showed a significant reduction of KYNA levels, associated with reduced ventral tegmental area dopamine firing activity [20], restoration of nicotine-evoked glutamatergic activity [4], and improved cognitive functions [19]. Consequently, the inhibition of hKAT2 could be a promising strategy in research on schizophrenia and related neurological and cognitive impairment.

For inhibition studies and structure-based inhibitor design, it is often necessary to obtain large amounts of pure active protein for high-throughput screening of lead compounds. In this paper we describe a speedy method of overexpressing hKAT2 using *Escherichia coli* expression system with optimized codons. The recombinant hKAT2 produced could be easily crystallized after using a single-step Ni-NTA based purification. A complete data set to 1.83 Å was collected and a structure arising from this would represent the highest reported resolution for hKAT2.

## 2. Materials and methods

### 2.1. Materials and instruments

Ampicillin, pyridoxal-5′-phosphate (PLP), isopropyl β-D-1-thiogalactopyranoside (IPTG), L-kynurenine, 2-ketoglutarate, imidazole, phosphate buffer saline (PBS), Tris, PEG-4000, Luria Broth (LB broth), lysozyme, DNase I, sodium citrate dihydrate, sodium chloride acetonitrile for HPLC, and protease inhibitor cocktail (for *E. coli* expressed protein purification) were all purchased from Sigma–Aldrich. The expression plasmid pET15b was purchased from Invitrogen. The gene sequence and sub-cloning procedure of the *hKAT2* were performed by Genscript. SDS running buffer, pre-made gels (12%), Laemmli sample buffer, Precision Plus Protein™ Dual Colour Standards, Coomassie blue stains and glass column were purchased from Bio-Rad. Ni-NTA resin was purchased from Qiagen. Amicon® ultra 4 mL centrifugal filter units with a 30 kDa nominal MW cutoff (NMWC) were purchased from Millipore. The HPLC detection was carried out using a CBM-20A Shimadzu HPLC system equipped with a 25 × 4.6 mm Ascentis® C18 HPLC column (10 μm particle size).

### 2.2. Bacterial strains

*E. coli (E. coli)* BL21 (DE3) and Rosetta 2.

### 2.3. Codon optimization of hKAT2 open reading frame (ORF)

The *hKAT2* ORF was optimized for *E. coli* expression via switching rare codons, GC content adjustment, removal of negative CIS elements and negative repeats using the online tool OPTIMIZER (http://genomes.urv.es/OPTIMIZER/) [21] in combination with OptimumGene™ (Genscript) and manual sequence adjustment (supplementary data).

### 2.4. Expression of recombinant hKAT2

Bacterial cell transformation was performed through a standard heat-shock procedure using chemically competent *E. coli* [BL21 (DE3)] or *E. coli* (Rosetta 2) cell lines. The transformed bacterial cells were grown on a media plate with 100 μg/mL ampicillin at 37 °C for 10 h, and one colony was used for overnight growth. The overnight cultures were used to inoculate 2.5 L of LB broth with 100 μg/mL ampicillin to an initial $OD_{600nm}$ of 0.1. The protein expression was induced by adding IPTG to a final concentration of 0.1 mM to cell cultures in mid-log phase ($OD_{600nm}$ of 0.6–0.8). After induction, the cell cultures were incubated at 15 °C under vigorous shaking for 24 h. Bacterial pellets were harvested by centrifuging the cultures at 11,000 × *g* at 4 °C for 30 min and stored at −80 °C for subsequent use.

### 2.5. Purification of recombinant hKAT2

The purification of recombinant hKAT2 was developed using a rapid Ni-NTA-affinity chromatography method [22]. First, the bacterial pellets harvested from 2.5 L culture (approximately 4 g) were resuspended in 40 mL of buffer A (20 mM Tris–HCl, pH8.0, 50 mM NaCl) containing 40 μM pyridoxal-5′-phosphate (PLP), 37.5 mM imidazole and protease inhibitor cocktail. The resuspended culture were treated with lysozyme and DNase I on ice for 20 min and then sonicated to facilitate cell lysis and centrifuged at 11,000 × *g* for 30 min at 4 °C. The soluble fraction of the culture was incubated with 5 mL of Ni-NTA resin equilibrated with 10 column volumes of buffer A with 37.5 mM imidazole for 30 min at 4 °C. After washing extensively with 10 column volumes of buffer A with 50 mM imidazole, 3 × 10 mL elutions containing purified protein were collected in buffer A with 200 mM imidazole. The purified recombinant hKAT2 was then concentrated using an Amicon® centrifugal filter unit (30 kDa NMWC). The protein concentration was estimated using a NanoDrop ND-1000 Spectrophotometer at $Abs_{280nm}$ (1 Abs = 1 mg/mL) [23]. The identity and purity of the protein samples were determined using sodium dodecyl sulphate gel electrophoresis (SDS-PAGE).

### 2.6. Activity assay of recombinant hKAT2

The enzyme activity assay for hKAT2 was developed using a slightly modified procedure of an existing method [24]. A 50 μL reaction mixture containing 5 mM of L-kynurenine, 5 mM of 2-ketoglutarate, 40 μM PLP and 0.9 μg purified hKAT2 in 10 mM phosphate buffer (pH7.5) was used to determine the activity of the recombinant protein. The reaction mixture was incubated at 37 °C for 10 min and then an equal volume of 0.8 M formic acid was added to terminate the reaction. A control was prepared in an identical fashion using the same reagents but without enzyme. After centrifugation reaction mixture at 18,000 × *g* at 4 °C for 10 min the supernatants were taken and diluted for 40 times for HPLC analysis. Both KYN and KYNA peaks were detected at 330 nm using a C18 reverse-phase column using 7% (v/v) acetonitrile and 93% (v/v) water as the mobile phase.

### 2.7. Crystallization of recombinant hKAT2

The hKAT2 crystals were obtained by hanging-drop vapour diffusion method based on a published protocol [18]. A 24-well tissue plate was used for the crystal growth. Each well contained 1 mL of reservoir solution comprised of 100 mM sodium citrate, pH5.6, 200 mM NaCl, and 24% PEG-4000. To 1 μL protein solution at 16 mg/mL or 7 mg/mL in buffer A with 200 mM imidazole, 1 μL reservoir solution was added and mixed on a siliconized coverslip that was used to seal the reservoir. Crystals appeared in the hanging drop after approximately 1 week at 20 °C.

### 2.8. X-ray data diffraction collection from crystal

The hKAT2 crystals (0.3 × 0.4 × 0.8 mm) were removed from the droplet using a LithoLoop (loop size 0.1 mm) and immediately flash frozen in liquid nitrogen. X-ray diffraction data were collected under a continuous cryo-nitrogen stream at 100 K using MX2