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Expression, purification and crystallization of GSK3β in complex with the flavonoid morin

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GSK3β is an important kinase that functions in cellular signaling pathways. Morin, a flavonoid that is plentiful in nature, was found as an inhibitor of GSK3β that can reduce tau pathology *in vivo* and *in vitro*. To identify how morin inhibits GSK3β, GSK3β protein was overexpressed and purified using affinity and ion exchange chromatography. GSK3β protein was crystallized with morin using hanging drop vapor diffusion method in the presence of 18 % (v/v) PEG 4000, 100 mM sodium citrate (pH 6.5), and 5 % (v/v) 2-propanol at 290 K. X-ray diffraction data was collected to a maximum resolution of 2.14 Å. The crystal belonged to $P2_1$, with unit cell parameters a = 67.6 Å, b = 134.4 Å, c = 100.4 Å, $\alpha = \gamma = 90.0°$, $\beta = 103.8°$.

INTRODUCTION

Glycogen synthase kinase 3β (GSK3β) is a proline-directed Ser/Thr protein kinase that acts as a key regulator of various cellular signaling pathways, including the Wnt, Hedgehog, Notch, and inflammation (Ali et al, 2001; Martin et al, 2005). GSK3β is also involved energy metabolism, neuronal cell development and body pattern formation (Plyte et al, 1992).

Alzheimer's disease (AD) is a chronic neurodegenerative disease which is the cause of dementia. Recent research has found that GSK3β is related to the pathogenesis of AD through amyloid beta (Aβ), phosphorylated tau and mitochondrial dysfunction (Reddy, 2013). Increased production of Aβ, converted from β-Amyloid precursor protein (APP) by presenilin, results in deposition into plaque in the extracellular region, and leads to the pathogenic hyperphosphorylation of tau by increasing the activity of GSK3β (Hooper, 2008).

Morin (3,5,7,2',4'-pentahydroxyflavone) (Figure 1) is a flavonoid that can protect cells against damage from oxygen radicals. Morin was originally isolated from mulberry figs and heartwood of old fustic (*Chlorophora tinctoria*), and it is rich in many oriental medicinal herbs (Morris et al, 1951); thus, it can be isolated from nature. Morin has many physiological effects, including anti-cancer effects (Chung et al, 2016 ; Zhang et al, 2018). Morin was previously reported to directly inhibit GSK3β, resulting in blocking GSK3β-induced tau phosphorylation *in vitro* and *in vivo* (Gong et al, 2011). However, it remains unknown how morin inhibits GSK3β at the atomic level. In this work, we describe the expression, purification, crystallization and X-ray crystallographic analysis of GSK3β complex with morin.

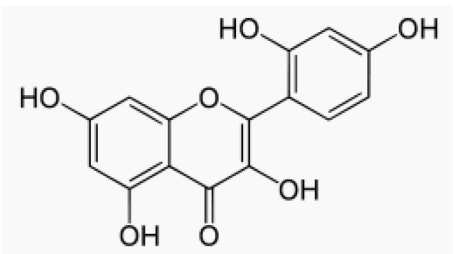

FIGURE 1 | Structure of morin. Chemical structure of morin (3,5,7,2',4'-pentahydroxyflavone).

RESULTS AND DISCUSSION

GSK3β was purified to evident homogeneity by nickel-affinity chromatography followed by ion-exchange chromatography. The elution volume of GSK3β was appeared at 150 mM NaCl and confirmed the purified protein showed single band using SDS-PAGE (Figure 2).

The GSK3β protein was crystallized with morin 18 % (v/v) PEG 4000, 100 mM sodium citrate (pH 6.5), and 5 % (v/v) 2-propanol (Figure 3). The crystals were transferred to cryoprotectant solution composed of the corresponding conditions described above and 20% ethylene glycol, and quickly frozen by liquid nitrogen. X-ray diffraction data were collected to a resolution of 2.14 Å on beamline 5C at the Pohang Accelarator Laboratory (PAL, Pohang, Korea) (Figure 4). The crystals of GSK3β with morin complex belonged to the space group P21 with unit cell parameters a = 67.6 Å, b = 134.4 Å, c = 100.4 Å, $\alpha = \gamma = 90.0°$, β

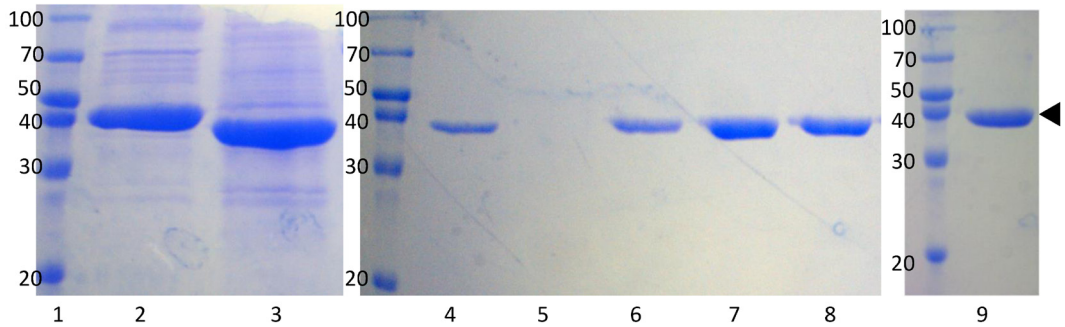

FIGURE 2 | Purification of GSK3β. SDS-PAGE of purification of recombinant GSK3β. Lane 1 shows molecular weight markers (labelled in kDa). Lane 1-9 show the purification procedure of GSK3β using Ni-NTA and Ion exchange chromatography. Lane 2, elution with 500 mM Imidazole; lane 3, after TEV cleavage; lane 4, sample after using desalting column; lane 5, flow through from Resource S column; lanes 6-8, elution with 150 mM NaCl; lane 9; final sample after full purification. Purified GSK3β is indicated with an arrow.

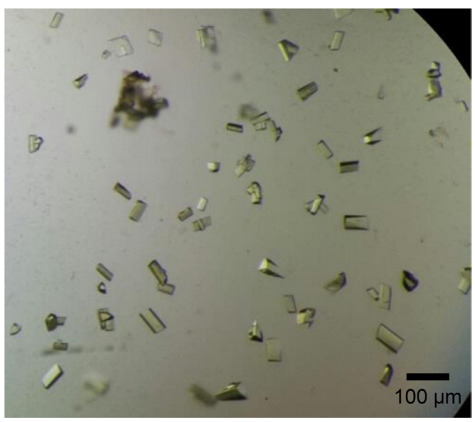

FIGURE 3 | Crystals of GSK3β in complex with the flavonoid, morin. Crystals of the best quality were produced from condition 18 % (v/v) PEG 4000, 100 mM sodium citrate (pH 6.5), and 5% (v/v) 2-propanol and grew to maximum dimensions of 0.1 x 0.1 x 0.2 mm within 3 days.

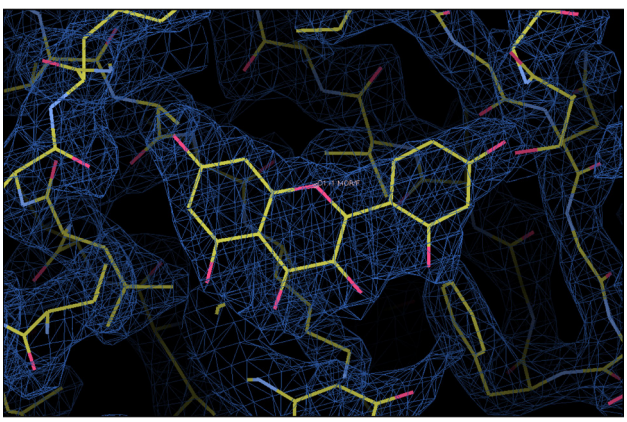

FIGURE 4 | Electron density map of Morin in GSK3β. Morin is well fitted in electron density map.

= 103.8°.

The crystal structure was solved by molecular replacement using human GSK3β, PDB code 1I09 (Harr et al, 2001) as the search model in the program MOLREP (Vagin et al, 1997). Coot was used for the visualization of the electron density maps and the manual rebuilding of the atomic model (Emsley et al, 2004). The model was refined using the program REFMAC5 (Vagin et al, 2004) at a 2.14 Å resolution. The initial electron density map, which was good quality with backbone atoms well defined by electron density, allowed us to build a three-dimensional GSK3β complex structure with morin. Crystallographic model building and refinement of the structure of 2.14 Å resolution are in progress.

METHODS

Expression of GSK3β

The expression and purification of the mouse GSK3β kinase domain have been previously described (Kim et al, 2015). Mouse GSK3β (residues 14-420) was ligated into the pFASTBAC-HTA vector (Invitrogen, USA) to generate the recombinant baculovirus for the Bac-to-Bac baculovirus expression system (Invitrogen). The resulting construct had an appended His6-affinity purification tag and a cleavage site for the TEV protease to GSK3β sequence. According to the manufacturer's instructions, recombinant baculovirus was constructed. The protein was expressed after infection of the virus at a multiplicity of infection of 5 in 2.0 × 10^6 SF9 cells in CCM3 media (Hyclone, USA). The cells were harvested by centrifugation and stored at -80°C until use.

Purification of GSK3β

The cells were thawed in a buffer consisting of 20 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM imidazole, 3 mM β-mercaptoethanol, and 10% glycerol and disrupted by homogenization (100 strokes). The cell lysate was applied to Ni-NTA metal affinity chromatography, and then, the His6-tagged protein was pooled and incubated with the recombinant TEV protease overnight to cleave the His6 tag. After changing the buffer by a desalting column in a buffer containing 20 mM HEPES (pH 7.5), 50 mM NaCl, and 1 mM DTT, Resource S column (GE Healthcare, USA) was applied using a linear gradient of 0.05 to 1 M NaCl in 20 mM HEPES (pH 7.5). The GSK3β protein was eluted at 150 mM NaCl, and the fractions were pooled. The final protein sample was concentrated to 5 mg/mL using a centrifugal concentrator (Millipore, USA).

Crystallization of GSK3β in complex with the flavonoid, morin

Prior to the crystallization, morin (Sigma, USA) was incubated at 5 mM

TABLE 1 | Crystallization

Method	Hanging-drop vapor diffusion
Plate type	SPL plate (24-well)
Temperature (K)	290
Protein concentration (mg ml^{-1})	5
Buffer composition of protein solution	20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM DTT
Composition of reservoir solution	18 % (v/v) PEG 4000, 100 mM sodium citrate (pH 6.5), and 5 % (v/v) 2-propanol
Volume and ratio of drop	1.5 μl; 1:1 ratio of protein and reservoir solutions
Volume of reservoir (ml)	0.5

TABLE 2 | Data collection and processing

Diffraction source	Beamline 5C, PAL
Wavelength (Å)	0.978
Temperature (K)	100
Detector	Pilatus 6M
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	$P2_1$
a, b, c (Å)	67.6, 134.4, 100.4
α, β, γ (°)	90.0, 103.8, 90.0
Resolution range (Å)	40.0-2.14 (2.18-2.14)*
Total No. of reflections	88,879
Completeness (%)	97.8 (95.0)*
R_{mer} (%)	7.6 (34.9)*
$I / \sigma I$	38.2 (3.8)*
Redundancy	6.0 (4.3)*

*The numbers in parentheses are statistics for the highest-resolution shell.

final concentration in the GSK3β protein sample (5 mg/ml) for 30 min at 4°C. Co-crystallization of the GSK3β protein with morin was performed using the hanging drop vapor diffusion technique. The plate crystals were observed at 17°C under a condition optimized for hanging drop experiments by mixing 1.5 μL of the protein sample with the same volume of the reservoir solution containing 18 % (v/v) PEG 4000, 100 mM sodium citrate (pH 6.5), and 5 % (v/v) 2-propanol. Crystallization information is summarized in Table 1.

X-ray Diffraction of GSK3β in complex with the flavonoid, morin

Diffraction data were collected from single crystals flash-frozen at 100 K in a nitrogen stream. The dataset was measured to a 2.14 Å resolution using a beamline 5C on a Pohang accelerator laboratory synchrotron with X-rays at a 1.0 Å wavelength (Park et al, 2017). The data were auto-indexed and processed with the HKL suite (Otwinowski et al, 1997). The data statistics are summarized in Table 2.

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

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